BACILLUS SUBTILIS EXTRACYTOPLASMIC FUNCTION SIGMA FACTORS CONTROL ANTIBIOTIC PRODUCTION AND RESISTANCE

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BACILLUS SUBTILIS ECF SIGMA FACTORS CONTROL ANTIBIOTIC PRODUCTION AND RESISTANCE

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Bacillus subtilis, the model organism for Gram-positive bacteria, expresses seven extracytoplasmic function (ECF) σ factors (called σ^M, σ^W, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC}). σ^M, σ^W, σ^X, and σ^V are well-characterized and mediate cell envelope stress responses. The functions of σ^Y, σ^Z and σ^{YlaC} remain largely unknown. One challenge in understanding the regulatory roles of ECF σ factor is that they display significant redundancy in their regulons.

In this study, we investigate the regulation of *B. subtilis* antibiotic production and resistance by ECF σ factors using both global analytical techniques (cDNA microarrays, phenotypic arrays, and transposon mutagenesis) and classical genetic and physiological tests. This work began with the observation that a triple *sigMWX* mutant (strain ΔMWX) lost its ability to inhibit the growth of other bacilli strains. We present evidence that deletions of σ^X and σ^M underlie this phenotype and that in wild type cells these ECF σ factors activate the expression of a transcription factor Abh. Abh stimulates the production of a peptide antibiotic called sublancin, which is able to inhibit the growth of other bacilli. We also compared the transcriptomic profiles and phenotypic traits of strains lacking these three σ factors (ΔMWX) and all 7 ECF σ factors (Δ7ECF) with a wild type strain. Deletion of all 7 ECF σ factors affects the

transcriptions of over 80 genes, most of which are regulated by σ^M, σ^W, or σ^X. The ΔMWX and Δ7ECF strains are more sensitive to several cell envelope disrupting compounds when compared to wild type including two β-lactam antibiotics (aztreonam and cefuroxime). Finally, we investigated the ECF σ factor-dependent genetic and biochemical mechanisms that mediate resistance to cefuroxime. σ^M is the major determinant in cefuroxime resistance with σ^X playing a smaller role. These σ factors regulate at least three pathways involving the regulatory proteins Abh and Spx, and a cyclic-di-AMP synthase DisA. Collectively, the data in this work suggests that ECF σ factors regulate an intricate regulatory network that contributes to both antibiotic production and resistance in *B. subtilis*.

BIOGRAPHICAL SKETCH

Yun Luo was born on October 12th, 1981 in Bobai, GuangXi, China. Growing up in a small town and a close family, she always wanted to see "the world".

After graduating from high school, Yun applied for a college she knew nothing about, and majored in her favorite subject, Biology. After receiving her Bachelor degree from Xiamen University, FuJian, China, she went on to pursue a Master degree with Dr. Volker Brözelin South Dakota State University, studying the multiple cellular behavior of *Bacillus cereus* growing in soil. During this time she found her passion for microbiology and genetics. With the support of Dr. Brözel, she applied to all the top research universities she could think of, and Cornell surprised her with an admission offer.

In September 2006, Yun began her graduate study at Cornell University, Ithaca, NY, a place half a globe away from home. In the following year, she joined the group of Dr. John Helmann. She was fascinated by the regulation of ECF σ factors, and enjoyed working in this friendly and supportive environment. This dissertation summarizes the major findings of her study.

To my parents and grandma

ACKNOWLEDGMENTS

I truly enjoyed my six years of Ph.D. study in Cornell, and I would like to thank all the people who helped me along the way for their support and good memories.

I am sincerely grateful to my supervisor and mentor, Dr. John Helmann, who has supported and guided me through my graduate study. When it comes to advising, John has the perfect balance in keeping me on the right track, yet allowing room for me to think and explore independently. His incredible knowledge and passion in science, hard work ethic, and true kindness have shown me not only how to think and work as a scientist, but also how to be a better person. I would also like to thank to my minor advisors Dr. Joe Peters (Genetics) and Dr. Linda Nicholson (Biochemistry) for their advice, availability, and encouragement.

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INTRODUCTION

1.1 Bacillus subtilis and its cell envelope structure and synthesis

Bacillus subtilis, a ubiquitous soil bacterium, has a typical Gram-positive cell envelope structure: a cell membrane surrounded by a thick peptidoglycan (PG) layer and associated anionic polymers (39, 113) (Figure 1.1). The cell envelope plays a vital role for cell growth. It maintains cell shape, counteracts turgor pressure, and transports molecules in and out of the cell (54, 135). It is thereby a prime target for antibiotics (137).

The cell membrane of *B. subtilis* consists of anionic phospholipids (phosphatidylglycerol and cardiolipin), neutral lipids (glycolipids and phosphatidylethanolamine), and a small percentage of a positively charged phospholipid (lysyl-phosphatidylglycerol) (27,30,117). It has a net negative charge and is therefore a target for cationic antimicrobial peptides (CAMP). *B. subtilis* also contains teichoic acids (TA) that are either membrane-associated (lipoteichoic acid, LTA) or PG-bound (wall teichoic acid, WTA) (Figure 1.1). LTA and WTA are individually dispensable but collectively these anionic polymers are essential (39). There are few antibiotics known to target TA biosynthesis, but a small molecule targocil was recently discovered to inhibit the growth of Gram-positive *Staphylococcus aureus* by sequestering the WTA transporter TagGH (111, 124).

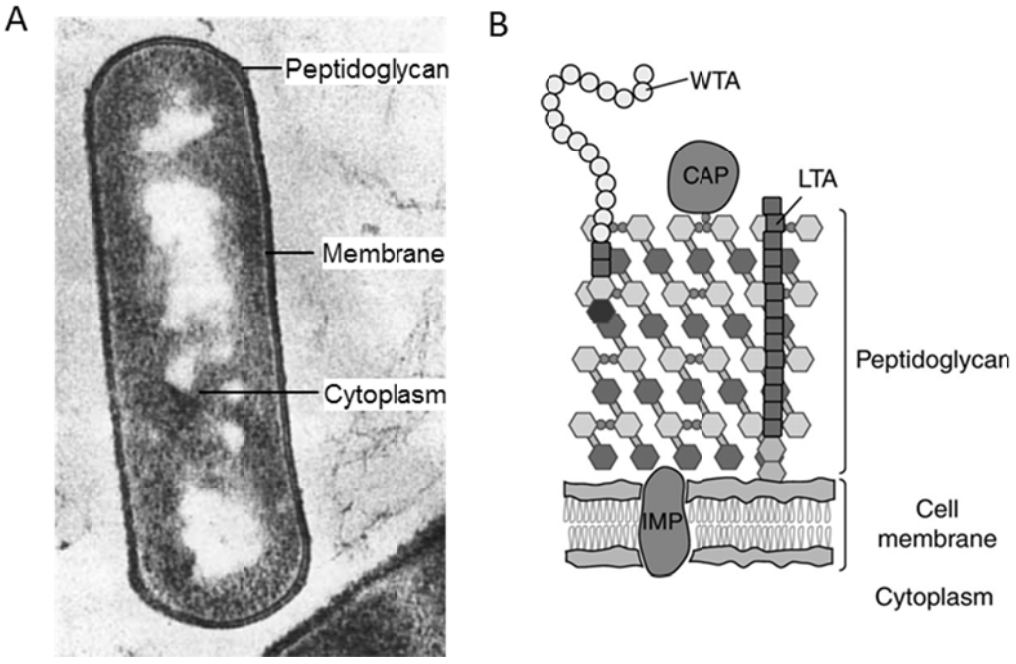


Figure 1.1. *Bacillus subtilis* cell and its cell envelope structure. **(A)** Electron micrograph of a *B. subtilis* cell. Adapted from (33). **(B)** Illustration of Gram-positive cell envelope structure. CAP, covalently attached protein; IMP, integral membrane protein; LTA, lipoteichoic acid; WTA, wall teichoic acid. Adapted from (113).

The PG layer is often referred to as the cell wall in *B. subtilis*. It is a network of glycan strands consisting of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), that are cross-linked by peptide side chains. PG biosynthesis starts with a stepwise conversion of Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) to Uridine diphosphate-N-acetylmuramic acid (UDP-MurNAc)-pentapeptide (Figure 1.2). In *B.subtilis*, the pentapeptide consists of L-Ala-D-Glu-mDAP-D-Ala-D-Ala. The UDP-MurNAc)-pentapeptide is then covalently bonded with the membrane-bound undecaprenyl-phosphate to form Lipid I. GlcNAc is attached to Lipid I, producing Lipid II. Lipid II is translocated to the extracytoplasmic face, where the GlcNAc-MurNAc-pentapeptide is incorporated into the nascent PG strand by the activity of transglycosylase (TG). Concurrent with or soon after polymerization, the pentapeptide side chain is cross-linked by transpeptidase (TP). The cross-linking peptide bond is usually formed between the 3rd (mDAP) and the 4th (D-Ala) amino acids in the adjacent PG strand sidechains. Both TG and TP are activities of high molecular weight penicillin binding protein (HMW PBP), and they are the targets of moenomycin and β-lactam antibiotics, respectively. Many of the PG synthesis enzymes and intermediate products are also targets of antibiotic action (Figure 1.2). The glycan strand adopts a right-handed helix-like conformation, with a periodicity of three disaccharide units (per turn of the helix), and the peptide side chains extend radially from the glycan strand. Many (but not all) side chains of adjacent glycan of the strands are cross-linked, creating a three-dimensional, stress bearing meshwork of strands (10, 39, 83).The degree of cross-linking varies in different strains and growth conditions. About 29-33% of PG side chains are cross-

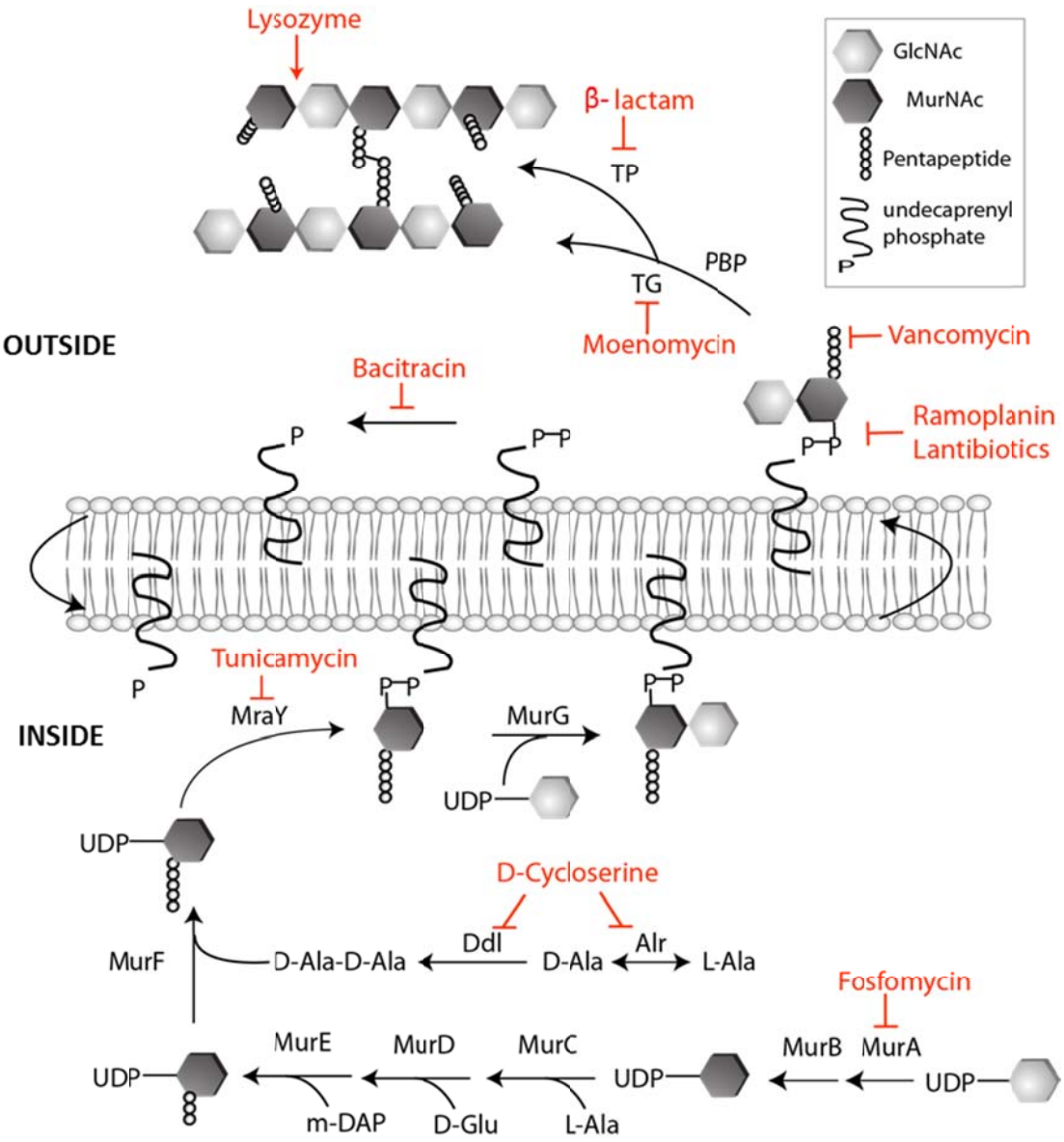


Figure 1.2. A schematic representation of peptidoglycan biosynthesis in *B. subtilis*. Some cell wall antibiotics relevant to this study are shown in red and their target sites are indicated by "—|". Lysozyme hydrolyzes the β-1, 4-glycosidic bond between MurNAc and GlcNAc, and is indicated by the arrow. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; m-DAP, *meso*-diaminopimelic acid; TG, transglycosylase; TP, transpeptidase; PBP, penicillin binding protein.

linked in the vegetative cells of *B. subtilis* strain 168 (39).

PG is also a dynamic polymer structure: glycan strands and peptide cross-links can be cleaved by PG hydrolases (autolysins) in order to allow PG maturation and cell expansion. In addition, autolysins are important for a variety of cellular functions, such as protein secretion, motility, chemotaxis, competence, and sporulation (11, 74, 109, 115). There are 35 autolysin genes (annotated and putative) found in the genome of *B. subtilis*. Together they specifically hydrolyze almost all the different bonds in PG (115, 136). The activities of autolysins are tightly regulated, and their de-regulation can cause cellular autolysis.

The biosynthesis and maturation of PG do not occur as isolated events. Instead, they are mainly organized by two sets of cytoskeletal machineries: one is scaffolded by the actin-like protein MreB, and is primarily dedicated to the lateral cell wall synthesis; the other is coupled with tubulin-like protein FtsZ at the mid cell and is involved in cell division (Figure 1.3). Multiple studies using fluorescence microscopy suggest that MreB forms one continuous helical filament along the length of the cell (23, 24, 28, 31, 61, 64, 128). MreB and its homologs (Mbl and MreBH) recruit and interact with, directly or indirectly, proteins involved in cell shape determination (e.g. RodA, MreC, MreD), PG biosynthesis and hydrolysis (e.g. Mur, PBP1, PBP2a, PbpH, and LytE) and TA biosynthesis (e.g. TagU and TagT), and thereby organize PG and TA synthesis in the lateral cell wall. However, recent works using high resolution imaging techniques proposed a different model (32, 42, 133, 134, 140): MreB and its associated proteins form discrete patches, instead of a long helical filament. This

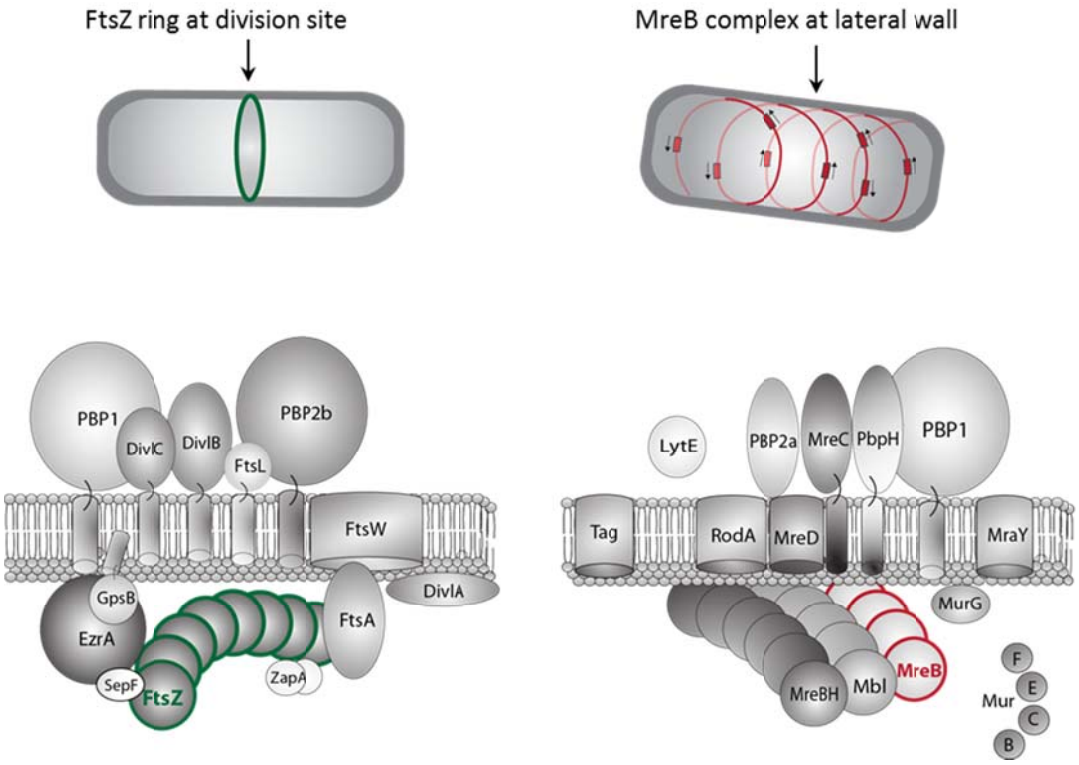


Figure 1.3. Peptidoglycan synthesis at the division site and the lateral cell wall in *B. subtilis*. **(A)** Schematic models for the FtsZ ring (green) at the cell division site (Left), and MreB complex (red) at the lateral cell wall (Right). The MreB complex was originally observed as a continuous helix, but it was recently shown to form discrete patches. Arrows next to the patches indicate their moving directions. **(B)** Schematic models for the components of the FtsZ complex (Left) and MreB complex (Right). Adapted from (24, 41, 140).

MreB complex moves circumferentially around the long axis of the cell while organizing PG synthesis on-site. This movement is bidirectional, and is driven by PG synthesis. FtsZ, on the other hand, forms a constricting ring at mid cell and recruits over a dozen of proteins to the divisome (Figure 1.3). These proteins carry out the functions of preseptal elongation, septum formation and cell separation (37, 102).

1.2 Antibiotics target at the cell wall

1.2.1 Cell wall biosynthesis as antibiotic target pathway

Because of its crucial role, the cell wall is a prime target for antibiotics (114, 137) (Figure 1.2). These antibiotics can either directly inactivate the biosynthetic enzymatic activity or sequester required substrates. Here, a selection of antibiotics related to the studies in the later chapters will be discussed in more detail, with emphasis on the mode of action and the resistance mechanisms employed by bacteria.

β-lactam antibiotics

β-lactam antibiotics are characterized by the presence of a β-lactam ring (Figure 1.4**).** Since the historical discovery of penicillin in the soil fungus *Penicillium chrysogenum* by Fleming in 1929, a variety of β-lactam antibiotics have been discovered and developed. They are now the most widely used antibiotics in medicine (38, 71). β-lactam antibiotics usually target the penicillin binding domain of PBPs, which are

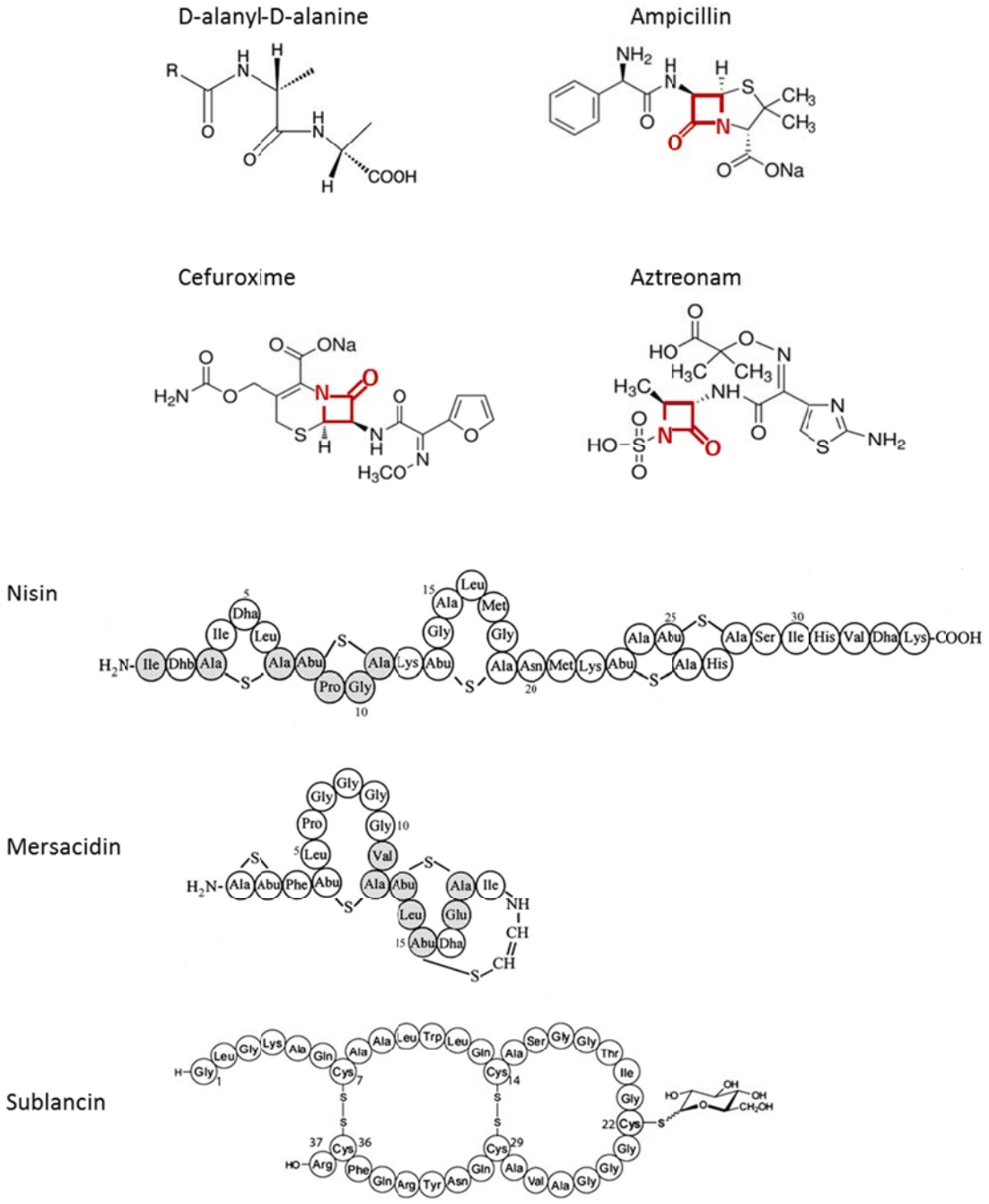


Figure 1.4. Structures of D-alanyl-D-alanine, β-lactams and peptide antibiotics. The β-lactam rings are highlighted in red. Amino acids that are important for the activity of nisin and mersacidin are shaded. Ala-S-Ala, lanthionine; Abu-S-Ala, β-methyllanthionine; Dha, didehydroalanine; Dhb, didehydrobutyrine. Adapted from (16, 89).

transpeptidases or carboxypeptidases involved in PG cross-linking and maturation (Figure 1.2). β-lactam structurally mimics the D-Ala-D-Ala dipeptide substrate of PBP (79) (Figure 1.4). A serine residue at the catalytic domain of PBP attacks the carbonyl carbon atom of the β-lactam ring, forming a stable acyl-enzyme complex. This β-lactam-enzyme complex is hydrolyzed at a very slow rate, effectively preventing further transpeptidation reactions (110, 146).

Based on studies using the Gram-positive genera *Staphylococcus* and *Streptococcus,* and the Gram-negative species *Escherichia coli* and *Pseudomonas* spp., the major resistance mechanisms towards β-lactams are believed to be the following: (i) expression of β-lactamase(s) that inactivate the antibiotics; (ii) expression of mutated or mosaic PBP alleles that have low affinity for β-lactams; and (iii) the expression of a β-lactam specific pump (97, 143). However, these three mechanisms cannot be applied or found in the Gram-positive *B. subtilis*. In chapter 4, we found that there are multiple stress response pathways that can contribute the intrinsic resistance to β-lactam antibiotics in *B. subtilis*.

Bacteriocins: lantibiotics and sublancin

Most antibiotics are natural products (and their derivatives) isolated from soil microorganisms (137). The antimicrobial peptides produced in bacteria are often referred to as bacteriocins. They are typically potent narrow spectrum antibiotics directed primarily against closely related bacteria.

Lantibiotics (lanthionine containing antibiotics) is a major group of bacteriocins. They are typical CAMPs containing amphiphilic or hydrophobic regions, and hence preferentially interact with the negatively charged cell membrane (25, 144). The best studied lantibiotics are nisin (produced by *Lactococcus lactis*) and mersacidin (produced by *Bacillus spp.* strain HIL Y-85, 54728) (Figure 1.4). Nisin and nisin-like molecules such as epidermin (produced by *Staphylococcus epidermidis*) and subtilin (produced by *B. subtilis* strain ATCC6633) interact with the pyrophosphate of Lipid II, and form a lantibiotic-Lipid II complex (Figure 1.2). These complexes aggregate and generate defined pores in the membrane, resulting in the leakage of low molecular weight compounds (e.g. K+, H+, phosphate), the dissipation of proton motive force (PMF), and eventually cell death (15, 57, 93). Mersacidin and related lantibiotics bind to both the MurNAc-GlcNAc sugar moiety and the pyrophosphate of Lipid II (14), thereby blocking the incorporation of the disaccharide units into PG. Mersacidin and most of the mersacidin-like lantibiotics, except plantaricin C, do not form pores at the membrane (141).

Sublancin 168 is a peptide antibiotic produced by *B. subtilis* strain 168. It is encoded by the *sunA-sunT-bdbA-sunS-bdbB* operon located in a prophage called SPβ. This operon encodes genes for sublancin pre-peptide synthesis, modification and transportation. Although originally classified as a lantibiotic (91), sublancin was recently identified as a glycopeptide with an unusual S-linked glucose attached to a cysteine residue (Cys22) (89) (Figure 1.4). The mode of action for sublancin remains unknown, but it is speculated to form pores in a similar manner as nisin. In addition, Kouwen *et al*. (2009) showed that the inhibition of sublancin relies on the presence of

a large mechanosensitive ion channel MscL, suggesting that MscL may serve as a direct target at the membrane or as a gate of entry to the cytoplasm (72).

Immunity to bacteriocins in the producer strain is often provided by its cognate immunity genes, which typically encode ABC transporters and/or immunity proteins (2). For example, nisin immunity is provided by NisFEG and NisI. NisFEG encode an ABC transporter system that exports nisin. NisI is a membrane-bound protein with high affinity to nisin, which may sequester nisin, and hence lower the nisin concentration and reduce its binding to substrate Lipid II (119, 125). Immunity to sublancin is provided by a membrane protein SunI (34). When the glycosylated Cys22 residue of sublancin was mutated to serine, the producer strain lost its immunity and became sensitive to this mutated product, suggesting that the S-glycoside moiety of sublancin may be involved in the immunity recognition with SunI (138). The detailed immunity mechanism of SunI protein still requires further investigation.

In non-producer strains, the major bacteriocin resistance mechanisms are to limit the substrate availability, and to express homologs of immunity genes. For nisin resistance, cells reduce Lipid II accessibility by modulating membrane negative charges. These modifications include coupling the positively charged D-alanine to teichoic acid (TA) and increasing the amount of neutral lipid phosphatidylethanolamine at the membrane (13). In *B. subtilis,* these modifications are mediated by σ^X, an extracytoplasmic function σ factor (ECF σ factors are discussed in section 1.4.2). In addition, The presence of divalent cations (e.g. Mg^{2+} and Ca^{2+}) also facilitate resistance to nisin, presumably by stabilizing the cell membrane and

preventing nisin from reaching the membrane (26). In the case of sublancin, its resistance is mediated by the ECF σ factor σ^W in *B. subtilis*. This σ factor regulates the expression of resistance genes, some of which appear to be homologous to immunity genes (18).

1.2.2 The consequences of antibiotic inhibition

Although the mechanisms of antibiotic-target interactions have been studied in great detail, the consequence of this primary interaction at the cellular level is not well understood, and remains a subject of investigation. Based on their inhibitory effects, antibiotics are generally classified as bactericidal drugs that kill bacteria, and bacteriostatic drugs that only inhibit growth (92). Almost all of the cell wall inhibitors are bactericidal antibiotics. For example, the inhibition of PBP transpeptidase by β-lactam causes cell lysis and cell death. Years of research have been focused on this lytic effect, and suggest that it is due to increased autolysin activity. Indeed, autolysin-deficient bacterial strains isolated from either clinical or research laboratories showed dramatically reduced rates of cell lysis and increased tolerance to β-lactam antibiotics (49, 100, 101, 130). How β-lactams trigger autolysin activity is still not known but is presumed to occur through PBP interaction. Several mechanisms have been proposed including an increase in turgor pressure due to continuous cell growth with a non-expanding cell wall (69), the accumulation of un-crosslinked PG substrate, and the depolarization of the membrane potential (94).

Autolysin activity is only one aspect of the story. Strains of *Streptococcus pneumonia* and *B. subtilis* lacking autolysins can still be killed by β-lactams or bacteriocins, suggesting that there is a lysis-independent mode of killing (73, 86, 131). Recent work from Kohanski *et al*. (2007), proposed a common mechanism for bactericidal antibiotics (not bacteriostatic antibiotics) which is to stimulate hydroxyl radical ($^{\bullet}$OH) formation. They observed a transient depletion of NADH upon antibiotic treatment in both *E. coli* and *S. aureus*. This was followed by a depletion of iron from iron-sulfur clusters and stimulation of internal Fenton reactions, resulting in the generation of $^{\bullet}$OH (70). $^{\bullet}$OH is a highly reactive oxygen species (ROS) that introduces oxidative damage to proteins, lipids, and DNA. There is no known enzyme to detoxify $^{\bullet}$OH, making it extremely toxic (58).

In *Bacillus* spp., there are several lines of indirect evidence suggesting that ROS are formed upon antibiotic treatment. In *B. subtilis*, a burst of free radicals was detected upon exposure to bactericidal antibiotics such as kanamycin, a protein synthesis inhibitor (85). Exposure to the cell wall synthesis inhibitors enduracidin and bacitracin induces the expression of numerous Spx-regulated genes (107). Spx is a transcriptional regulator that is known to protect cells from ROS stress by inducing cytoplasmic thiol production (149). In addition, NO and H_2S were also found to protect cells from ROS damage imposed by antibiotic (e.g. the β-lactam cefuroxime) exposure in *Bacillus anthracis, S. aureus, Pseudomonas aeruginosa* and *E. coli* (46, 112). Although some key aspects of antibiotic-ROS induced cell death were identified, the exact pathways and targets involved in $^{\bullet}$OH formation remain to be elucidated.

1.2.3 Antibiotics as signaling molecules

Although commonly applied in high doses in medicine, antibiotics may have roles other than inhibiting cell growth, especially since their concentrations rarely reach high levels in their natural environment (predominantly soil). It has been proposed that antibiotics function as signaling molecules for intra- and inter-species communication (6, 29, 43, 104).

At sub-lethal concentrations, antibiotics can modulate global gene expression, stimulate cell growth and/or trigger cell differentiation. Tobramycin, an aminoglycoside produced by the soil bacterium *Streptomyces tenebrarius,* was shown to induce biofilm formation in *P. aeruginosa* (52, 75). Similarly, imipenem (a variant of the β-lactam thienamycin produced by *Streptomyces flavogriseus*) induces the expression of β-lactamase and promotes the accumulation of alginate, the major component of the biofilm matrix in *P. aeruginosa* (6). A biofilm is a multicellular community attached to a surface and encased in an extracellular matrix, where the matrix functions as a physical and biochemical barrier to exclude antibiotics (53). Its formation may constitute a response to antibiotic stress.

With regard to intra-species communication, the lantibiotics nisin, subtilin, and epidermin have been shown to function as quorum sensing autoinducers and control their own biosynthesis in strains of *L. lactis*, *B. subtilis*, and *S. epidermidis*, respectively (66, 68). Given the complexity of the soil environment, it is attractive to think that antibiotics may have dual activities for signaling within producer strain

populations, and for inhibiting competitors. These activities might help cells compete for resources and space.

1.3 ECF σ factors and their structure, function and regulation

Inhibition of cell wall biosynthesis by antibiotics often triggers stress responses inside the cell. These responses allow cells to express key proteins which help minimize the damage. In Gram-positive bacteria, the cell envelope stress response (CESR) is often mediated by two component systems (TCS) and extracytoplasmic function (ECF) σ factors (62). Here, I will focus on ECF σ factors.

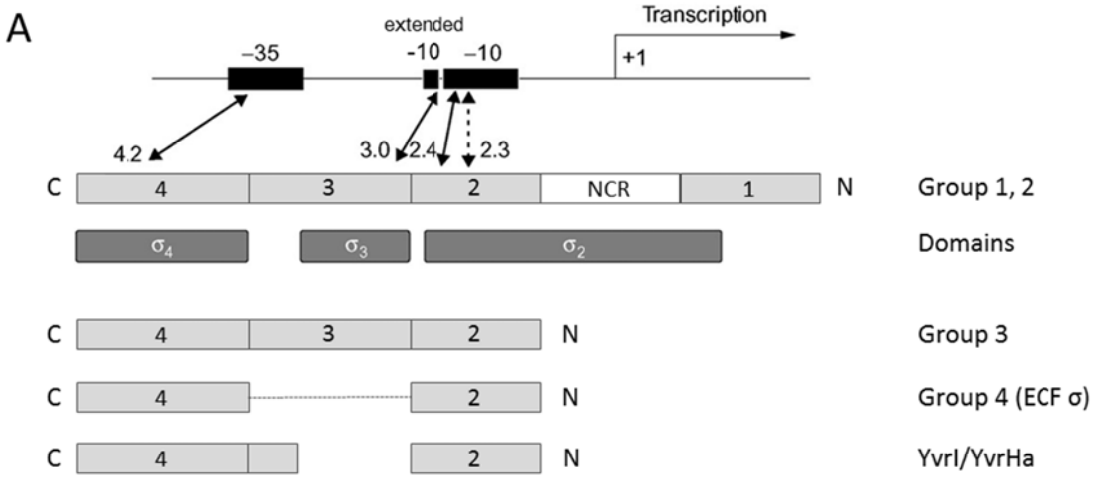
1.3.1 σ factors and ECF σ factors

Bacterial RNA polymerase (RNAP) holoenzyme consists of a five subunit core enzyme ($\alpha_2\beta\beta'\omega$) and a dissociable σ factor (87). The core enzyme is the minimal assembly for the catalytic activity of transcription, and σ factors determine promoter specificity and stabilize open complex formation in promoter DNA (87). All bacteria express one primary σ factor and most bacteria also harbor a variable number of alternative σ factors. The primary σ factor is involved in the expression of house-keeping genes while each alternative σ factor usually activates transcription of a specific set of genes in response to environmental cues. Together, σ factors regulate gene expression in the cell (7, 48).

The bacterial σ factors are divided into two structurally and functionally distinct families, σ^{70} and σ^{54}, both of which are named after the prototypes found in *E. coli* (84, 90, 99). *B. subtilis* encodes one σ^{54}-like factor (σ^{L}) and at least 17 known σ^{70}-like factors. The σ^{70}-like factors can be further divided into 4 groups based on their primary sequence conservation (47, 76) (Figure 1.5**)**. The group 4 σ factors are also referred to as extracytoplasmic function (ECF) σ factors, for they often regulate cell surface-associated functions such as those related to secretion, transport and extracytoplasmic stress (77). These σ factors only retain σ regions 2 and 4, which the most conserved regions of all σ factors. These two regions constitute the minimally required domains for binding to core RNAP and interacting with the -10 and -35 motifs of promoter elements (Figure 1.5).

The numbers of ECF σ factors in bacteria varies greatly, ranging from zero to as many as 83. A high number of ECF σ factors is often associated with a high number of TCS and generally reflects the complexity of the environment in which the host lives. Bacteria living in a stable condition (e.g. some endosymbionts *Chlamydia* spp.) typically have a small genome and no ECF σ factors. Species living in a diverse and variable environment such as soil harbor a relatively high number of ECF σ factors. For example, there are 7 ECF σ factors in *B. subtilis*, 19 in *P. aeruginosa*, 50-60 in *Streptomyces coelicolor*, and 83 in *Sorangium celluosum* (116).

Figure 1.5. Structural features of σ^{70} and the interaction between RNAP and promoter DNA. **(A)** The primary sequence of σ^{70} can be divided into four conserved regions (regions 1 through 4). These regions can be further divided into sub-regions. The interactions between σ sub-regions and the -35 and -10 elements of a promoter are indicated by double arrows. Three structural domains (σ_2, σ_3 and σ_4, dark grey) are indicated underneath the linear sequence structure. These domains coordinate closely (not precisely) with regions assigned by sequence comparisons. σ factor groups 1 and 2 contain all 4 sequence regions, group 3 contains regions 2-4, and group 4 (ECF σ factor) contains regions 2 and 4. YvrI and YvrHa constitute a two-component σ factor. YvrI contains regions of a canonical region 4 and a highly divergent region 2. YvrHa contains a σ region 2. NCR, non-conserved region; N, N-terminus; C, C-terminus. **(B)** A schematic illustration for the relative position and function of the major domains of an σ factor (dark grey) with the RNAP core and promoter DNA. The arrow indicates the direction of transcription. Adapted from (90, 145).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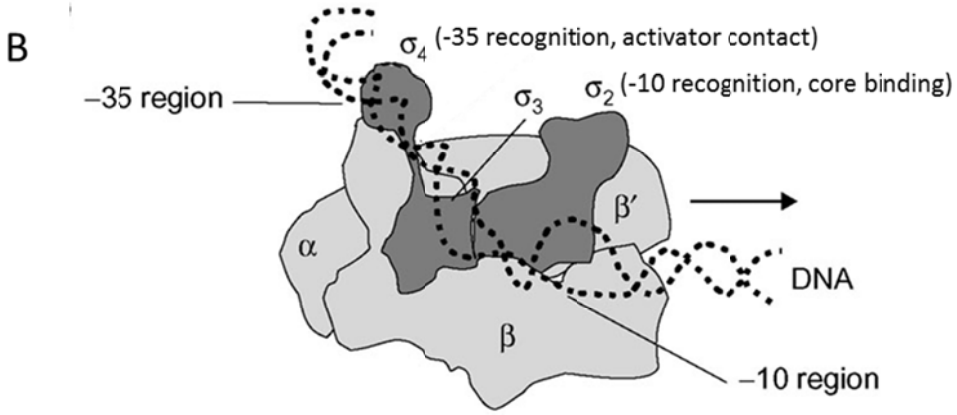
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1.3.2 The Regulation of ECF σ factors

As alternative σ factors, ECF σ factors compete with the primary σ factor for RNAP core, and redirect RNAP to new promoter sites. Their expression and activity are therefore tightly regulated. Studies on the regulatory systems of several ECF σ factors, including *E. coli* σ^E and FecI, *B. sutilis* σ^W, *P. aeruginosa* $\sigma^{AlgT/U}$, and *S. coelicolor* σ^R, have suggested a few common features of these systems (47, 116) (Figure 1.6). An ECF σ factor operon often encodes the σ factor and a corresponding anti-σ factor. In the absence of a stimulus, the anti-σ factor sequesters and inactivates its cognate ECF σ factor through protein-protein interactions. In the presence of an external signal, the anti-σ factor is inactivated by proteolysis, conformational change, or another mechanism, and releases the ECF σ factor. The σ factor is then free to bind to RNAP core and direct transcription of its own operon (autoregulation) and other target genes. However, there are some exceptions. For example, σ^Z in *B. subtilis* is not known to be regulated by an anti-σ factor, or to autoregulate its own expression (4). σ^E in *S. coelicolor* is activated by a two component system CseCB (56). In *Mycobacterium tuberculosis*, σ^J and σ^I are regulated through a transcriptional cascade, where the activation of σ^J can induce the expression of σ^I (55).

1.4 ECF σ factors and cell envelope stress response

B. subtilis encodes 7 ECF σ factors, namely σ^M, σ^W, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC} (Figure 1.6). The inducing conditions and physiological roles of four of the σ factors (σ^M, σ^W, σ^X, and σ^V) have been well characterized (35, 45, 47, 60). They respond cell-envelope

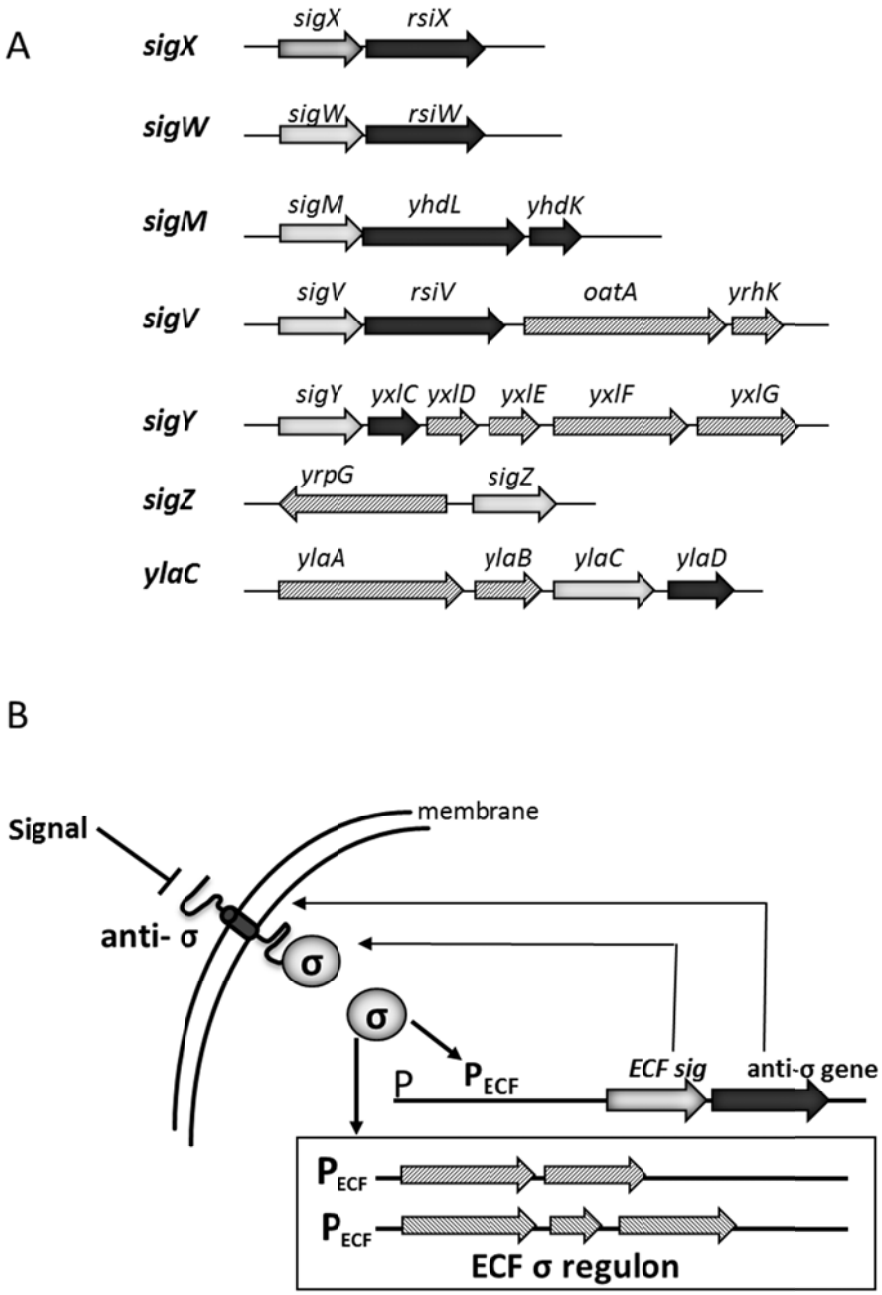

Figure 1.6. ECF σ operons and their regulation in *B. subtilis.* **(A)** Gene organization of ECF σ operons in *B. subtilis*. ECF σ genes are shown as grey arrows, and anti-σ genes as black arrows. Genes located in the same or adjacent operons, and regulated by the nearby ECF σ factors are shown as arrows filled with stripes. **(B)** A schematic representation of a typical ECF o factor expression pattern. ECF σ factors usually autoregulate their own operons, and other genes which may be distributed around the chromosome. Adapted from (47).

disrupting compounds, and activate the expression of large sets of genes. Studies on the other three σ factors (σ^Y, σ^Z and YlaC) are ongoing. σ^Y is weakly induced by nitrogen starvation (129). It is only know to directly regulate its own operon, and one gene *ybgB* (encoding a membrane protein with unknown function). YlaC is induced in response to hydrogen peroxide (108). The inducing conditions for σ^Z remain unknown.

1.4.1. The best defense is a good offense: ECF σ factors control antibiotic production

As described above, most antibiotics are secondary metabolites of soil microorganisms (i.e. they are produced through non-essential, secondary pathways that are dispensable in many growth conditions) (106). Under nutrient limiting or other stressful growth conditions, expression of these metabolites may provide survival advantages, ranging from inhibiting competitors to controlling cell growth and differentiation (104, 106) (discussed in section 1.2.3).

Strains of *B. subtilis* can produce over two dozen antibiotics (bacteriocins) collectively, most of which are strain specific (1, 118). The common lab strain 168 is capable of producing seven bacteriocins, including five ribosomally synthesized peptide antibiotics: sublancin (91), subtilosin A (5, 148), the SdpC sporulation delay protein (44), the SkfA killing factor (3, 44), and the TasA protein (121), and two non-ribosomally synthesized antibiotics: the phospholipid bacilysocin (126), and the dipeptide bacilysin (59, 65). *B. subtilis* 168 also possesses synthase genes for surfactants (surfactin and fengycin) and polyketides, but they are not produced due to a frame-shift mutation in the gene encoding the phosphopantetheine transferase Sfp.

Sfp is required to activate the surfactin and fengycin synthetase, as well as polyketide synthase (118). This is likely due to mutations accumulated from the mutagenesis work with X-rays in the mid-1940s and a long history of laboratory cultivation (17, 147). As a result of this history, numerous genetic and phenotypic differences exist between domestic lab strains.

The expression of bacteriocins is controlled by multiple regulatory pathways. The transcription of the *sunA* operon (encoding sublancin) is repressed by a pleiotropic transcriptional regulator AbrB. This repression is relieved by phosphorylated Spo0A which inhibits *abrB* transcription upon entering stationary phase (122). In Chapter 2, we present evidence that ECF σ factors σ^X (mainly) and σ^M (to a lesser extent) can activate the expression of a third transcriptional regulator and an AbrB homolog, Abh. Abh competes with AbrB for binding at the *sunA* promoter, resulting in increased sublancin production (78, 123). The activation of *abh* transcription through σ^X ultimately increases the expression of the *yqxM-sipW-tasA* operon (88). TasA is not only a bacteriocin that is capable of inhibiting both Gram-positive and negative bacteria (121), but also a major protein component of biofilm matrix (12). TasA proteins form amyloid fibers that are essential for the integrity of the matrix (103). These fibers are anchored to the cell wall by YqxM (TapA), encoded by the first gene of the operon (105).

σ^Y was recently reported to be required for maintaining the SPβ prophage that harbors the *sunA* operon in a *B. subtilis* 168 derivative strain called JH642 (82), although we did not observe the loss of SPβ in our 168 strain when *sigY* is deleted.

Finally, σ^M is known to regulate the expression of a phospholipase (YtpA) that is required for bacilysocin synthesis (35, 126).

1.4.2 The defense: ECF σ factor contributes to antibiotic resistance.

ECF σ factors and their regulons can also directly defend against antibiotic stress through the enzymatic inactivation of antibiotics or modification of the drug targets. The individual regulons of σ^M, σ^W, σ^X, and σ^V, although partially overlapped, contribute uniquely to these ends (45, 47).

σ^X was one of the first ECF σ factors to be studied in detail. A *sigX* mutant is prone to cell lysis and sensitive to nisin and other CAMPs (20) The σ^X regulon includes two key operons (*dltABCDE* and *pssA-ybfM-psd*). The Dlt proteins incorporate positively charged D-alanine into TA (95). PssA and Psd catalyze the synthesis of the neutral cytoplasmic membrane lipid phosphatidylethanolamine. The incorporation of both positively charged TA and neutral lipids reduces the net negative charge of the membrane, thereby contributing to CAMP resistance and the prevention of cellular autolysis (20).

σ^W is perhaps the best studied ECF σ factor in *B. subtilis*. It is induced by several cell wall-acting antibiotics including fosfomycin, bacitracin, vancomycin, cephalosporin C, sublancin, and the toxic peptide SdpC (18, 22, 96). It is also induced by detergents (e.g. Triton-X-114, SDS) (22) and alkali stress (142). The activation of σ^W stimulates "antibiosis" regulons that provide resistance to some inducers such as fosfomycin, sublancin and SdpC (47). Fosfomycin is an inhibitor of MurA, the enzyme that catalyzes the first committed step in PG synthesis (63) (Figure 1.2). σ^W

activates the expression of *fosB*, a bacillithiol-S-transferase that enzymatically inactivates fosfomycin (19, 40). Together fosmycin and σ^W form an efficient feed-back loop, where the presence of fosfomycin induces σ^W, and σ^W mediates the detoxification of fosfomycin. Both sublancin and SdpC are bacteriocins produced by *B. subtilis*, and their immunities are provided by the immunity gene products of *sunI* (34) and *sdpI* (36), respectively. In strains lacking *sunI* or *sdpI*, the role of σ^W in resistance becomes apparent due to its regulation at three operons: the *yqeZ yqfAB* operon (sublancin resistance), and the *yknWXYZ* and *yfhLM* operons (SdpC resistance) (18). The functions of these operons are unknown, but *yknWXYZ* encodes a putative ABC transporter and *yfhL* encodes an SdpI paralog. YqeZ is a putative membrane-bound protease, and YqfAB are putative NefD-like flotillins. In addition, σ^W also contributes to the resistance to detergents and antimicrobial compounds produced by other bacilli (e.g. *Bacillus amyloliquefaciens* and *Bacillus atrophaeus*). This is in part due to the activity of a σ^W-dependent promoter located within the gene *fabHa*. Transcription initiated from this promoter reduces expression of *fabHa* and increases the transcription of downstream gene *fabF*, resulting in modulation in membrane fatty acid composition and a reduction in membrane fluidity (67).

σ^M is induced by bacitracin, vancomycin, moenomycin, and rhamnolipid (a biosurfactant produced by *P. aeruginosa*). The activation of σ^M confers resistance to bacitracin, moenomycin and rhamnolipid (35, 80, 127, 139). Bacitracin inhibits PG synthesis by binding to undecaprenyl pyrophosphate (UPP) and thereby preventing its recycling to the monophosphate form (120) (Figure 1.2). σ^M contributes to bacitracin resistance by upregulating the expression of *bcrC* (21, 81). BcrC is an UPP

phosphatase and is able to compete with bacitracin for the UPP substrate (9). The *bcrC* promoter is also recognized by σ^X and σ^I (21, 132). How σ^M contributes to moenomycin and rhamnolipid resistance is less clear, and the genes responsible have not yet been identified. However, ECF σ factors such as σ^M have the potential to modulate the structure of the entire cell envelope and thereby contribute to antibiotic resistance. Components of the lateral cell wall synthesis complex (MreBCD, RodA, PBP1), PG biosynthetic enzymes (MurB, MurF, Ddl), and divisome components (MinCD, DivIB) all harbor σ^M promoters (Figure 1.3). In addition, σ^M also regulates the transcriptional regulators Spx and Abh, which are involved in resistance to the β-lactam drug cefuroxime (Chapter 4).

σ^V is induced by and provides resistance to lysozyme (45, 51). Lysozyme is a muramidase that cleaves the β-1,4-glycosidic bond between MurNAc and GlcNAc (Figure 1.2). In addition, this positively charged enzyme also functions as a CAMP (50, 136). σ^V contributes to lysozyme resistance by regulating expression of the *dltABCDE* operon and gene *oatA*, the latter which is transcribed as part as the *sigV* operon (*sigV-rsiV-oatA-yrhK*). OatA is a MurNAc specific O-acetyltransferase and O-acetylation of MurNAc reduces the ability of lysozyme binding to its substrate (8). The D-alanylation at TA through the activity of *dlt* operons helps to repel lysozyme from cell envelope. In addition, PbpX, a low molecular weight PBP and a member of the σ^X and σ^V regulons, may also contribute to lysozyme resistance (51).

1.4.3 The overlapping regulation of ECF σ factors

One major challenge in studying the regulation by ECF σ factors is the significant overlap in their regulons. Of the 60 genes regulated by σ^W, ~20 can be recognized by σ^X, σ^M, or σ^V. Similarly, about 30 out of the ~60 gene σ^M regulon, about 20 of the ~30 gene comprising the σ^X regulon, and almost all of the ~30 genes in the σ^V regulon can also be activated by a subset of, or all of the other three σ factors. This large degree of regulatory redundancy is mainly due to the fact that these ECF σ factors recognize a highly conserved "AAC" motif in the −35 region and a "CGT" motif in the −10 region of their promoters (Figure 1.7) (45, 80, 116) .

Despite this pronounced redundancy, some promoters are exclusively recognized by a single ECF σ factor. Even for promoters that can be activated by multiple σ factors, there appear to be a preference for certain σ factors. For example, the transcription of *abh* is primarily driven by σ^X, and only weakly by σ^M (Chapter 2, (78)). The expression of *bcrC* largely depends on σ^M, and less on σ^X (21). The mechanism for this promoter selectivity is not yet clear, but it is likely derived from the interaction between promoter DNA nucleotides and amino acids of the σ proteins. One example is σ^W and σ^X and their interactions with the -10 element (98). σ^X recognizes the CGAC motif of -10 element, while σ^W recognizes CGTA. Both σ factors can recognize CGTC at this site (Figure 1.7). Altering the motifs in the -10 element can switch the promoter preference for either σ^X or σ^W. Similarly, promoters recognized by σ^{MWXV} appear to contain multiple T's downstream of the -35 element, while this feature is less prominent in promoters recognized by only σ^{MWX} (Figure 1.7).

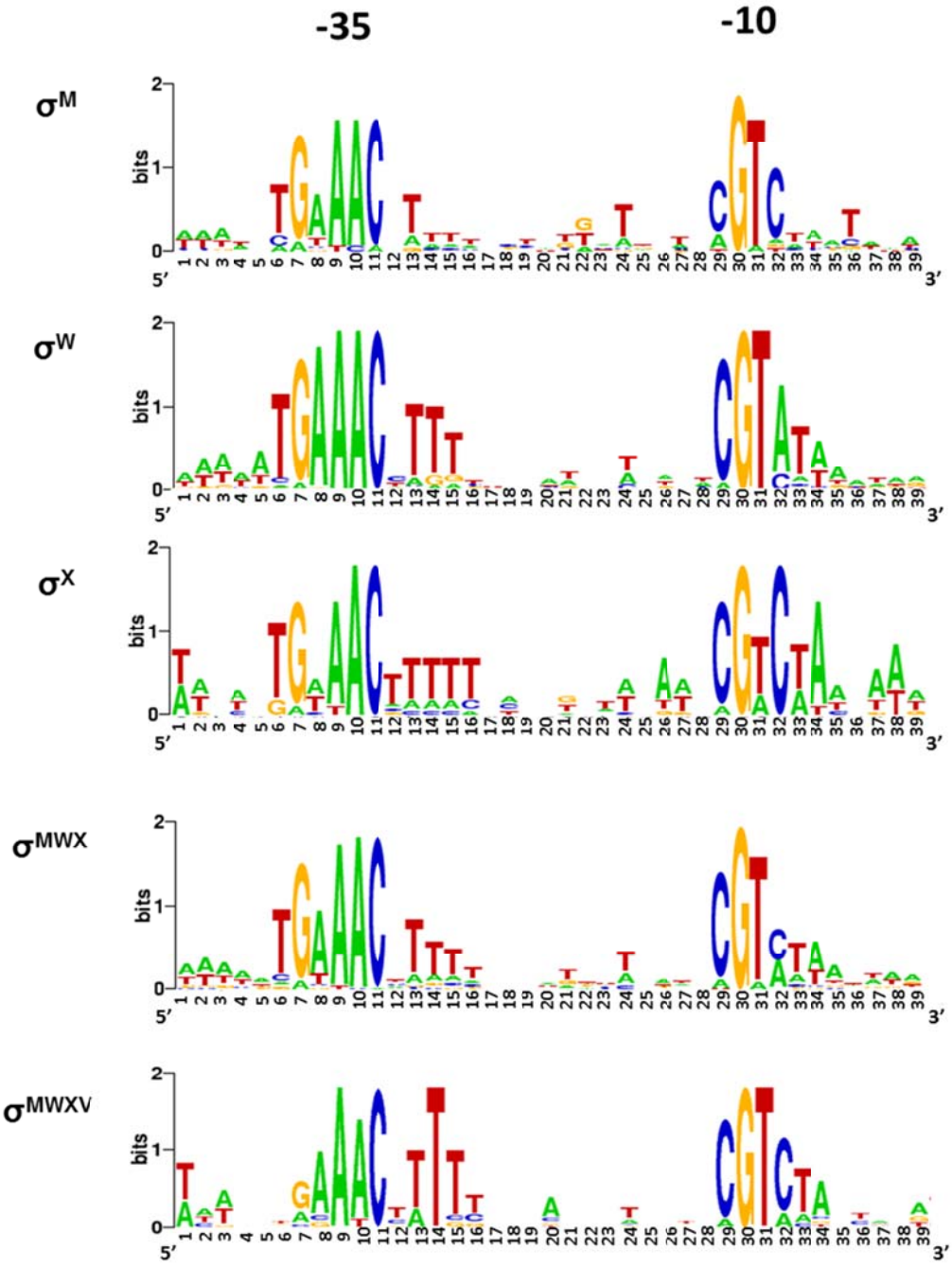

Figure 1.7. Promoter sequence consensuses recognized by ECF σ factors in *B. subtilis*. Each consensus was generated with promoter sequences that are recognized by either single or multiple ECF σ factors (as indicated) at the Weblogo server (http://weblogo.berkeley.edu/logo.cgi). The degree of sequence conservation is represented by the height of nucleotide symbols at each position. Adapted from (45, 80).

It is possible that these T's may facilitate the interaction between σ^V and the promoter DNA (45).

The regulatory redundancy of ECF σ factors complicates the studies on individual σ factors and their functions: the effect of a single ECF σ factor deletion is often masked by the expression of others. As a result, some phenotypes are only detectable when two or more ECF σ factors are mutated. For example, a triple mutant of *sigMWX* (strain ΔMWX) displays additional phenotypes not found in any of the single mutants. These phenotypes include increased susceptibility to several detergents and to cell wall-acting antibiotics ampicillin, D-cycloserine, and polymyxin B (80). When all 7 ECF σ factors were eliminated (strain Δ7ECF), additional phenotypes and transcriptional changes were also observed compared those in strain ΔMWX (Chapter 3). The overlapping regulation of ECF σ factors suggests a very intricate regulatory network, which could serve as a means to guarantee cell envelope integrity in a chemically-diverse environment.

1.5 Content of the dissertation

This dissertation focuses on the regulatory role of ECF σ factors in antibiotic production and resistance. In order to study the function of an individual ECF σ factor in an overlapping regulatory network, the major strategy employed in this work is to start with a multiple ECF σ mutant strain with a detectable phenotype, deduce the dominant σ factor by a process of elimination, and investigate the pertinent pathways that are regulated by the σ factor.

In chapter 2, research began with the observation that strain ΔMWX lost the ability to inhibit the growth of other bacilli strains as compared to the wild type strain. We showed that two ECF σ factors, σ^X and σ^M, are the relevant σ factors by testing all the possible combinations of single, double, and triple mutants of *sigMWX*. σ^X (mainly) and σ^M (to a less extent) regulate the expression of Abh, which directly activates the transcription of the *sunA* operon for sublancin production. Sublancin can inhibit the growth of nonlysogenic strains of *B. subtilis* and its close relatives *Bacillus pumilus*, *Bacillus licheniformis*, and *B. amyloliquefaciens*, as well as other Gram-positive bacteria, such as *S. aureus*. Therefore, it may provide competitive advantage in a diverse microbial environment.

Chapter 3 deals with the overlapping regulation of ECF σ factors. We acquired a strain harboring all 7 ECF σ null mutations (Δ7ECF) through collaboration with Kei Asai and Yoshito Sadaie (Saitama University, Japan). We compared the transcriptomic and phenotypic signatures associated with strains of Δ7ECF and ΔMWX. This comparison also allowed us to distinguish the functional contributions of the well-studied σ factors (*sigMWX*), and the lesser understood σ factors (*sigVYZ ylaC*). We found approximately 80 genes that at least partially depend on ECF σ factor for expression, most of which are regulated by σ^M, σ^W or σ^X. We also found new antibiotic sensitivity phenotypes associated with ECF σ factors, including two β-lactam drugs called aztreonam and cefuroxime. In addition, strain Δ7ECF showed greater reduction in biofilm formation than strain ΔMWX, suggesting a possible role of $\sigma^{YVZYlaC}$ in biofilm formation.

Chapter 4 considers the regulatory role of ECF σ factors in mediating cefuroxime resistance. σ^M is the major determinant and σ^X plays a secondary role. Using a Tn7 random mutagenesis-based analysis, we found that these two σ factors regulate three pathways involving Spx, Abh, and DisA. We propose that Spx contributes to drug resistance by antagonizing the ROS stress imposed by cefuroxime. Abh indirectly represses the expression of autolysin genes and thus inhibits cell lysis. DisA is a cyclic-di-AMP (c-di-AMP) synthase. c-di-AMP is essential for cell growth, and is involved in maintaining PG homeostasis.

In summary, the bacterial cell envelope is a vital cellular structure and a prime target for antibiotics. In *B. subtilis*, cell wall targeting antibiotics often induce the expression and activity of ECF σ factors, which activate a complex stress response. This ECF σ mediated response not only provides protection from antibiotics but also provides competitive advantages by regulating antibiotic production which can inhibit competitors in the soil environment.

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CHAPTER 2

EXTRACYTOPLASMIC FUNCTION SIGMA FACTORS WITH OVERLAPPING PROMOTER SPECIFICITY REGULATE SUBLANCIN PRODUCTION IN *BACILLUS SUBTILIS*

Bacillus subtilis harbors seven extracytoplasmic function (ECF) σ factors. At least three ECF σ factors (σ^M, σ^W and σ^X) are induced by, and provide resistance to, antibiotics and other agents eliciting cell envelope stress. Here, we report that ECF σ factors also contribute to antibiotic production. *B. subtilis* 168 strains that are lysogenic for the SPß bacteriophage produce sublancin which inhibits the growth of other, non-lysogenic strains. Genetic studies demonstrate that synthesis of sublancin is largely dependent on σ^X with a smaller contribution from σ^M. A *sigM sigX* double mutant is unable to produce sublancin. This defect is primarily due to the fact that the sublancin biosynthesis is positively activated by the transition state regulator and AbrB paralog Abh, which counteracts transcriptional repression of the sublancin biosynthesis operon by AbrB. Ectopic expression of *abh* bypasses the requirement for σ^M or σ^X in sublancin synthesis, as does an *abrB* mutation. In addition to their major role in regulating sublancin expression by activating *abh* transcription, σ^X and σ^M also have a second role as positive regulators of sublancin expression that is independent of AbrB and Abh. Since sublancin resistance in non-lysogens is largely dependent on σ^W, ECF σ factors control both sublancin production and resistance.

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2.1 Introduction

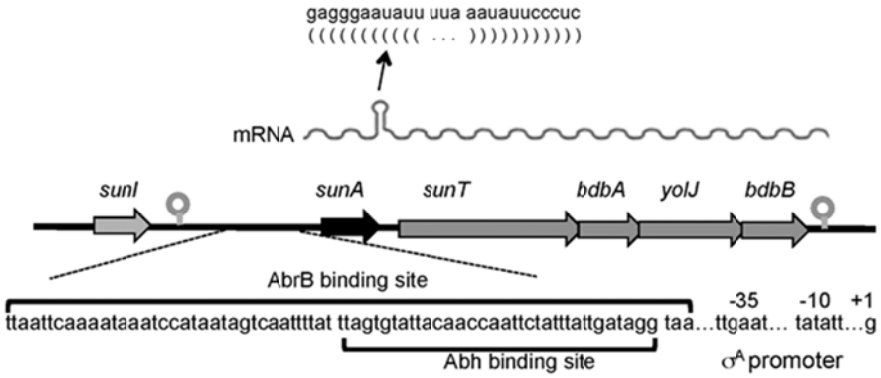
Bacillus subtilis, a ubiquitous soil bacterium, inhabits a fiercely competitive niche and devotes a large fraction of its genome to genes implicated in the synthesis of, and resistance to, antibacterial compounds. Regulation of antibiotic resistance functions is frequently mediated by extracytoplasmic function (ECF) σ factors which, as a class, control functions related to cell envelope and transport.

B subtilis harbors seven known ECF σ factors: σ^M, σ^W, σ^X, σ^Y, σ^Z,σ^V and σ^{YlaC} (17). Of these, the σ^M, σ^W and σ^X regulons are the best characterized. These three σ factors control overlapping sets of genes that are important for resistance against a variety of cell envelope-active compounds and antibiotics. In several cases, resistance genes are controlled predominantly by a single ECF σ factor. For example, σ^W controls genes that confer resistance to the peptidoglycan synthesis inhibitor fosfomycin (3), the toxic peptide SdpC, and the lantibiotic sublancin (2). The σ^M regulon includes a large number of operons implicated in cell wall synthesis and the corresponding *sigM* mutant is sensitive to several cell wall antibiotics including bacitracin, vancomycin and moenomycin. The σ^X regulon includes operons that modulate the net charge of both the cell membrane and cell wall and a *sigX* null mutant displays increased sensitivity to nisin and other cationic antimicrobial peptides (4, 5). In other cases, resistance to antimicrobial compounds appears to be dependent on gene(s) that are potentially expressed by more than one ECF σ factor. As a result, strains carrying multiple deletions in *sigM*, *sigW* and *sigX* show sensitivities to additional antibiotics such as D-cycloserine and some β-lactam antibiotics (21).

B.subtilis strains are known to produce more than two dozen antibiotics (31). In several cases, antibiotic biosynthesis and associated immunity functions are encoded on genomic islands or phage and are potentially transferred between closely related species by either transduction or natural competence. Sublancin 168 (hereafter called sublancin) is one of the bactericidal antibiotics produced by the reference strain, *B.subtilis* 168. Sublancin is classified as an unusual lantibiotic, with a β-methyllanthionine bridge and two disulfide bridges (26). Sublancin is extremely stable, and is active against gram-positive bacteria, including strains of *B. cereus*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. It inhibits both the outgrowth of endospores and vegetative cell growth. The mode of action has been speculated to involve pore formation in the cytoplasmic membrane. Kouwen *et al.* have recently found that a mechanosensitive channel of conductance MscL is required for sublancin susceptibility, but the precise mechanism remains unknown (20).

The sublancin operon is located in the prophage SPβ genome. This 4.5kb segment contains 5 genes: *sunA*, *sunT*, *bdbA*, *yolJ*, and *bdbB* (Figure 2.1A). All, except *bdbA,* are essential for sublancin production (8, 9). The structural gene *sunA* encodes presublancin, which is a 56-residue polypeptide possessing a 19-residue leader segment (26). *sunT* is located immediately after the *sunA* gene and encodes ABC-type transporter containing a proteolytic domain thought to cleave the leader peptide of sublancin during secretion across the membrane (9, 24). BdbB and BdbA comprise a thiol-disulfide oxidoreductase involved in the post-translational formation of disulfide bonds in sublancin (9). YolJ (SunS) contains a CxxS motif and it is also involved in the modification of sublancin (8, 33).

A



B

Spot / Lawn	168	Δ*sig MWX*	Δ*sunA*
B.pumilus BP1			
B.licheniformis ATCC8480			
B.amyloliquefaciens NRL B-14393			

Figure 2.1. (A) Schematic of the sublancin operon showing regulatory protein binding sites important for sublancin expression. AbrB and Abh binding sites are located upstream of a σ^A promoter. The *sunA* gene is, at least, co-transcribed with *sunT*, and a hairpin structure is found between *sunA* and *sunT*. The complementary sequence of the hairpin is indicated in a dot-bracket notation. (B) ECF σ factors regulate the synthesis of sublancin, which acts against other *Bacillus* spp. Strain 168, or the isogenic strain carrying a deletion of three σ factor genes (Δ*sigMWX),* or *sunA (*Δ*sunA)* were spotted onto lawns of 23 other *Bacillus* spp. strains. Only three representative lawns of *Bacillus* spp. are shown (see Table S2.1 for complete list). A clear zone is observed if the lawn strain is sensitive to the compounds produced by the spotted strains.

In this study, we show that the synthesis of sublancin requires the activity of either of two ECF σ factors, σ^M and σ^X. Genetic analyses establish that the influence of σ^M and σ^X results from their ability to activate transcription of *abh* which encodes a paralog of the pleiotropic transition state regulator protein AbrB. Both σ^M and σ^X can recognize an ECF σ factor-dependent promoter element preceding the *abh* gene and expression of *abh* from a heterologous promoter bypasses the requirement for either of these ECF σ factors. The function of Abh in activation of the *sunA* operon is to prevent repression by AbrB: *sunA* activity is constitutive in an *abrB abh* double mutant strain. Together with previous results, these findings establish that ECF σ factors control both the production of, and resistance to, antimicrobial compounds.

2.2 Material and Methods

Strains, plasmids and growth conditions. All *B. subtilis* strains, plasmids and oligonucleotides (oligos) used in this study are listed in Table 2.1. Bacteria were grown in liquid Luria–Bertani (LB) medium at 37°C with vigorous shaking, or on solid LB medium containing 1.5% Bacto Agar (Difco) with appropriate selection. Plasmids were amplified in *Escherichia coli* DH5α before transformed into *B. subtilis* strains. Ampicillin (amp, 100 μg ml^{-1}) was used to select *E.coli* strains harboring desired plasmids. For *B. subtilis*, antibiotics used for selection were as follows: spectinomycin (spc, 100 μg ml^{-1}), kanamycin (kan, 10 μg ml^{-1}), tetracycline (tet, 5 μg ml^{-1}), chloramphenicol (cat, 5 μg ml^{-1}), and macrolide-lincosoamide-streptogramin B (mls, contains 1 μg ml^{-1} erythromycin and 25 μg ml^{-1} lincomycin).

Table 2.1. Strains, plasmids and oligos used in this study.

Strains, Plasmids, or Oligos	Genotype, description	Construction, reference, or oligo sequence
***B.subtilis* strains**		
168	*trpC2*	Y.Sadaie (Saitama University, Japan)
CU1065	*trpC2 attSP*β	Lab strain
HB 5331	CU1065 *yqeZyqfAB::kan*	(2)
HB10016	168 *sigM::tet*	LFH-PCR→168
HB10103	168 *sigX::kan*	LFH-PCR→168
HB6164	CU1065 *sunA::kan*	(2)
HB10111	168 *sunA::kan*	HB6164 chrDNA→168
HB10113	168 *sigM::tet sigX::kan*	HB10103 chrDNA → HB10016
HB10131	168 *abh::spc*	LFH-PCR→168
HB10139	168 *abrB::tet*	LFH-PCR→168
HB10146	168 *abh::spc abrB::tet*	HB10139 chrDNA → HB10131
HB0020	CU1065 *sigW::mls*	(3)
HB10102	168 *sigW::mls*	HB0020 chrDNA →168
HB10107	168 *sigM::tet sigX::kan sigW::mls*	HB10102 chrDNA → HB10113
HB0911	CU1065 *sigV::cat*	(21)
HB10101	168 *sigV::cat*	HB0911 chrDNA →168
HB0009	CU1065 *sigY::mls*	(7)
HB10108	168 *sigY::mls*	HB0009 chrDNA →168
HB0032	CU10665 *sigZ::kan*	(6)
HB10109	168 *sigZ::kan*	HB0032 chrDNA →168
HB0029	CU1065 *ylaC::kan*	(6)
HB10110	168 *ylaC::kan*	HB0029 chrDNA →168
HB10182	168 *sunA::kan abh::spc*	HB10111 chrDNA →HB10131
HB10143	168 *sigM::tet sigX::kan abh::spc amyE::PsunA-lacZ (cat)*	HB10131 chrDNA, pYL18 →HB10113
HB10155	168 *sigM::kan sigX::spc abrB::tet amyE::P_{sunA}-lacZ(cat)*	HB10139 chrDNA, pYL18 →HB10113
HB10123	168 *lacA::P_{xylA}-abh(mls)*	pYL19 →168
HB10124	168 *sigX::kan lacA::P_{xylA}-abh(mls)*	HB10103 chrDNA→ HB10123
HB10128	168 *sigM::tet lacA::P_{xylA}-abh(mls) amyE::P_{sunA}-lacZ(cat)*	HB10016 chrDNA, pYL18→ HB10123
HB10137	168 *sigM::tet sigX::kan lacA::P_{xylA}-abh(mls) amyE::P_{sunA}-lacZ(cat)*	HB10103 chrDNA, pYL18→ HB10123
SWV121	*trpC2 pheA1 amyE::P_{abh}-lacZ(cat)*	M. Strauch (University of Maryland)
HB10147	168 *amyE::P_{abh}-lacZ(cat)*	SWV121 chrDNA → 168
HB10148	168 *sigM::tet amyE::Pabh-lacZ(cat)*	HB10147 chrDNA → HB10016
HB10149	168 *sigX::kan amyE::Pabh-lacZ(cat)*	HB10147 chrDNA → HB10103
HB10150	168 *sigM::tet, sigX::kan amyE:: Pabh–lacZ(cat)*	HB10147 chrDNA → HB10113

Table 2.1 (continued)

Plasmids		
pYL18	pDG1661-P_{sunA}-*lacZ* (*cat*)	This work
pYL19	pAX01- P_{xylA}-*abh* (*mls*)	This work
Oligos		
4195	sunA-for	ATGGAAAAGCTATTTAAAGAAG
4196	sunA-rev	TCTGCAGAATTGACGATAGT
4368	23S-RT-F	AAAGGCACAAGGGAGCTTGACTG CGAGA
4369	23S-RT-R	ATGAGCCGACATCGAGGTGCCAA ACCT
3314	AAP	GGCCACGCGTCGACTAGTACGGGI IGGGIIGGGIIG
4370	abh-rev-GSP1	GGCTTGAATTTCTTCGA
4371	abh-rev-GSP2	ACGCCATGAGGTTTGTACTT

Genetic techniques. Gene deletions were generated using long-flanking-homology polymerase chain reaction (LFH-PCR) as described (2, 22) with selection for the appropriate antibiotic resistance cassette. Chromosomal DNA transformations were performed as described (16).

Spot-on-lawn assays. Spot-on-lawn assays were performed as previously described (2). Briefly, lawn cells were grown to OD_{600} of 0.4 in LB, mixed 1:50 (culture: medium) with 2ml melted 0.7% or 1.5% LB agar, and poured into wells of an 8-well rectangular multidish (26mmx33mm, Nunc). Plates were dried for 30min in a laminar flow hood, and 2µl of the producer strain (OD_{600} of 0.6) was spotted in the center of the well. Plates were incubated at 37°C overnight (18h) before observation. Spot-on-lawn assays of each strain were performed in biological triplicates, and repeated at least three times. Several mutant strains harbor a reporter fusion *amyE::*P_{sunA}*-lacZ(cat)*. The presence of this reporter fusion did not influence the results of the spot-on-lawn assays.

β-galactosidase assays. Test strains carrying either an *abh* promoter-*lacZ* (P_{abh}-*lacZ*) fusion were grown overnight in LB medium containing appropriate antibiotics, and diluted 1:100 into 5ml LB medium. The cultures were incubated at 37°C with vigorous aeration, and sampled from logarithmic, transition, and stationary growth phases. β-galactosidase assays of each strain were performed in biological triplicate as described by Miller (25), and repeated at least three times. Data are reported as the mean and standard deviation.

RNA isolation, northern blotting, and slot blotting. Total RNA was extracted from 2 ml of cells grown to an OD_{600} of 0.4 (mid-log phase) using the RNeasy kit

(QIAGEN) according to the manufacturer's protocol. The RNA was quantified using Nanodrop spectrophotometer (NanoDrop Technologies) and visualized by agarose gel electrophoresis. Two DNA probes, *sunA* and 23rRNA were constructed by PCR with the primer pairs sunA-for (4195) and sunA-rev (4196), 23S-RT-F (4368) and 23S-RT-R (4369), respectively (Table 2.1). The probes were purified using the QIAGEN PCR purification kit and labeled with [γ-^{32}P] dATP (3,000 Ci/mmol, 10 mCi/µl; New England Nuclear) and T4 polynucleotide kinase (New England Biolabs). Unincorporated [γ-^{32}P] dATP was removed using a QIAGEN PCR purification kit. Northern blotting analysis was carried out by using the NorthernMax formaldehyde-based system (Ambion) following the manufacturer's instruction with 10 µg of total RNA and Zeta-Probe blotting membrane (Bio-Rad) in a downward transfer setup. The slot blotting was performed as described (29), where total RNA was transferred to Zeta-Probe blotting membrane using vacuum. The hybridization and washing steps for both Northern blot and slot blotting were performed with the NorthernMax hybridization kit according to the manufacturer's instruction. The blots were then wrapped in plastic wrap, exposed for 12h to a phosphor screen (Molecular Dynamics), and scanned using a Storm 840 PhosphorImager (Molecular Dynamics). The resulting images were analyzed with ImageQuant software (Molecular Dynamics).

5' rapid amplification of cDNA ends (5' RACE). The transcriptional start site of *abh* was determined using 5' RACE. Two µg of total RNA from mid-log phase culture were reversed transcribed to cDNA using TaqMan reverse transcription reagents (Roche) and oligo abh-rev-GSP1 (4370) as primer. The 3' end of cDNA was tailed with poly dCTP using terminal deoxynucleotidyl transferase (New England Biolabs).

The tailed cDNA were then amplified by PCR with primers AAP (3314) and abh-rev-GSP2 (4371). The PCR products were sequenced with Sanger sequencing technology.

2.3 Results and Discussion

ECF σ factors regulate antibiotic production. Previous studies have established that ECF σ factors, and in particular σ^W, plays a major role in the antibiotic-inducible expression of genes that provide resistance to bacteriocins and antibacterial peptides produced by other soil microorganisms including other *Bacillus* spp. (2). To determine whether ECF σ factors also contribute to antibiotic production, we tested the ability of *B. subtilis* strain 168 to inhibit the growth of other *Bacillus* spp. using a spot-on-lawn assay. In an initial screen, wild type (WT) strain 168 and its isogenic triple mutant lacking σ^M, σ^W, and σ^X (Δ*sigMWX*, HB10107) were used as spots, and 23 strains of *Bacillus* spp. (listed in Table S2.1) were used as lawn cells. Sixteen of the 23 strains showed less susceptibility to the triple σ factor mutant than to strain 168, while the other seven strains were similarly susceptible to both strains. Three representative stains that have differential susceptibility are shown in Figure 2.1B. In all cases, the inhibition zone sizes from Δ*sigMWX* spots were significantly reduced compared to those from strain 168. This result indicates that σ^X, σ^M, or σ^W, individually or in combination can regulate antimicrobial activity against other *Bacillus* spp. Notably, this antimicrobial activity was not observed using the 168 derivative strain CU1065 as the spotted strain (data not shown). Strain 168 differs from strain CU1065 in that it carries prophage SPβ, which encodes the lantibiotic sublancin 168 (26). To test

whether the killing effect of 168 cells was due to sublancin, a derivative of 168 lacking *sunA* (Δ*sunA*, HB10111) was spotted on lawns of the same 23 *Bacillus* spp. Deletion of *sunA* abolished the killing effect noted for all 16 strains that showed a differential susceptibility to 168 *versus* the Δ*sigMWX* strain (Figure 2.1B). This suggests that the product of the *sunA* gene (sublancin) is a major antibiotic active against other *Bacillus* spp. and is regulated either directly or indirectly by σ^X, σ^M, or σ^W.

σ^X or σ^M is essential for sublancin production. In order to identify which ECF σ factor(s) regulate sublancin production, more comprehensive spot-on-lawn assays were conducted using single, double, and triple ECF σ mutants as spots. The sublancin sensitive strain, strain CU1065, was used as the lawn strain. Since CU1065 lacks the SPβ prophage, it lacks both the ability to synthesize sublancin and the SPβ encoded immunity gene *sunI* (10), rendering it susceptible to killing by sublancin. As shown in Figure 2.2A (upper panel), we tested seven single ECF σ deletion mutant spots and only Δ*sigX* (HB10103) and Δ*sigM* (HB10016) strains showed reduced inhibition zone sizes compared to that from the WT spot. Judging from zone diameters, the Δ*sigX* strain impaired cell killing to a greater degree than did the Δ*sigM* strain. Deletions in *sigX* and *sigM* were additive as a Δ*sigMX* double deletion strain (HB10113) completely abolished the killing effect. This result indicates that σ^X and σ^M are the ECF σ factors that regulate sublancin production.

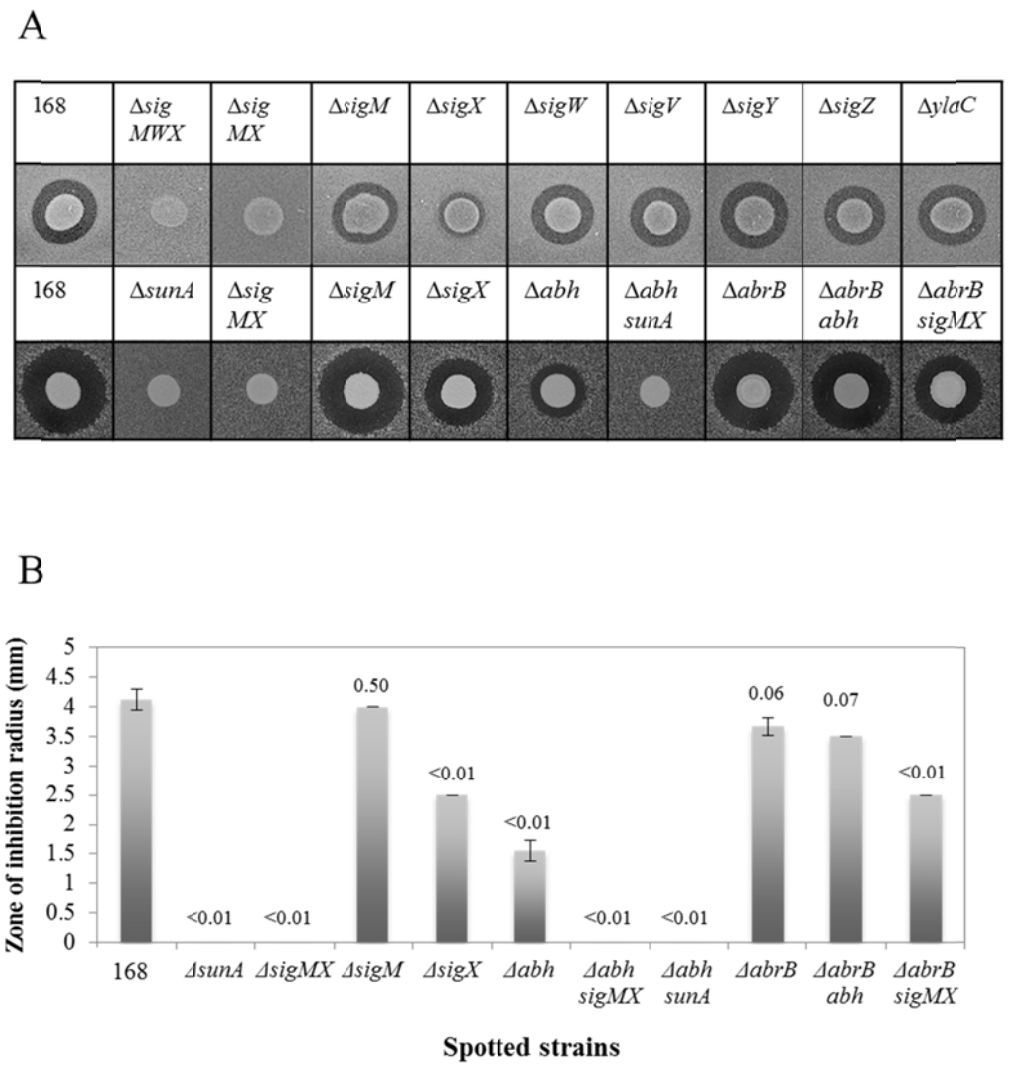


Figure 2.2. σ^X and σ^M regulate sublancin production. (A) Various deletion strains were spotted onto a *B.subtilis* CU1065 lawn (upper panel) and the hypersensitive strain HB5331 lawn (lower panel). (B) The radius of the zone of inhibition produced by various strains spotted on a lawn of HB5331. The radius was measured as the total diameter of the zone of clearing minus the diameter of the colony spot, divided by a factor of 2. The numbers above each bar are the P values for a paired Student T-test comparing 168 to mutants.

In order to confirm that *sigM* and *sigX* are strictly required for sublancin production, the Δ*sigMX* strain was spotted on a lawn of strain HB5331, a sublancin hypersensitive strain that harbors a *yqeZ-yqfAB* deletion in a CU1065 background (Figure 2.2A, lower panel). The *yqeZ-yqfAB* operon is expressed from a σ^W-dependent promoter and confers a modest level of resistance to sublancin in strains lacking SPβ (2). Even with this hypersensitive strain, no sublancin production (cell killing) was detected from a Δ*sigMX* double deletion spot (Figure 2.2A, lower panel), suggesting that in the absence of both of these σ factors, sublancin expression is effectively abolished.

Abh counteracts AbrB repression in sublancin synthesis. The region upstream of *sunA* harbors a consensus σ^A promoter sequence, but no apparent recognition sequences for either σ^X or σ^M (Figure 2.1A). This observation suggests an indirect regulatory role for σ^X and σ^M in activating *sunA* transcription. We therefore examined two additional regulators of sublancin transcription, Abh and AbrB, which bind overlapping sequences in the *sunA* promoter region. It has been proposed that AbrB is a repressor and Abh is an activator for *sunA*, but how they cooperate or compete to influence the overall regulation of *sunA* transcription remains unclear (32). In previous studies, an *abh* mutant was found to have an ~2-fold decrease in the level of expression from a *sunA-lacZ* reporter fusion (32) and we previously demonstrated that σ^X recognizes a promoter upstream of the *abh* gene (18). Thus, it seemed plausible that Abh might provide the link between ECF σ factor activity and activation of sublancin expression.

In our spot-on-lawn assays, when a strain carrying a deletion of *abh* (Δ*abh*, HB10131) was spotted onto the highly sensitive indicator strain HB5331, the inhibition zone was significantly reduced comparing to that from strain 168 (Fig. 2A, lower panel, and Fig. 2B), which confirms a positive role for Abh in sublancin production. However, when strains Δ*abrB* (HB10139) and Δ*abh abrB* (HB10146) were spotted onto the same lawn, the zones of inhibition were comparable to the wild-type strain 168. These results suggest that the role of Abh is to counteract repression mediated by AbrB: if AbrB repression is absent, Abh is not needed. This result contradicts the previous suggestion that Abh might function as a direct activator of sublancin expression since an *abh abrB* double mutant apparently failed to make an antimicrobial compound (presumed to be sublancin) as detected using *B. coagulens* as an indicator strain (32).

If the regulation of sublancin production by σ^X or σ^M is solely through Abh, one would predict that a Δ*abh* mutant would fail to make any detectable sublancin as also noted for the Δ*sigMX* double mutant in this bioassay. However, there is clearly residual antibacterial activity in the Δ*abh* strain (Figure 2.2). We first considered the possibility that the *abh* deletion might derepress synthesis of one or more antibiotics that are negatively regulated by Abh (32). However, the residual antibiotic activity in the Δ*abh* strain was eliminated in a Δ*abh sunA* double mutant (HB10182), indicating that this activity was due to sublancin synthesis (Figure 2.2). This raised the possibility that, in addition to their effects on Abh production, σ^X or σ^M may have a second pathway by which they activate sublancin synthesis. Consistent with this notion, deletion of *sigM* and *sigX* not only removes the residual activity detected in an

abh deletion strain, it also reduces the level of sublancin activity in an *abrB* deletion strain (Figure 2.2A, lower panel, and 2.2B). Together, these results suggest that σ^X or σ^M likely regulate sublancin production both by activation of Abh (which counteracts AbrB repression) and independent from Abh or AbrB.

σ^X or σ^M is required for transcription of *sunA*. To test whether the observed effects on sublancin production were due to regulation of *sunA* transcription, we measured mRNA levels using Northern blotting (Figure S2.1) and RNA slot blotting (Figure 2.3). The sublancin operon was previously found to be transcribed as two separate transcripts: *sunA* (0.2 kb) and *sunT-bdbA-yolJ-bdbB* (4.3 kb) (30). However, our Northern analysis detected a single major transcript of approximately 2~2.5kb using *sunA* as a probe (Figure S2.1). Our results suggest that *sunA* is, at least, co-transcribed with *sunT*. The downstream genes *bdbA*, *yolJ* and *bdbB* may also be co-transcribed with *sunAT*, since no gaps exist between *sunT-bdbA*, *bdbA-yolJ*, and *yolJ-bdbB*. This expected transcript of 4.5 kb was, however, not detected, which is possibly due to RNA processing. Interestingly, *sunA* gene itself is one of the most stable mRNAs in *B. subtilis* although no specific secondary structure related to mRNA stability was noted at its 5'end (15). We noted the presence of a potential hairpin in the 60 bp space between *sunA* and *sunT* (ΔG=-14.5 kcal/mol as determined using Mfold; (23, 34) (Figure 2.1A). It is possible that this 3'-end secondary structure protects *sunA* mRNA from degradation.

As expected based on the phenotype assays, *sunAT* mRNA levels were significantly reduced in the Δ*sigX,* Δ*sigMX* and Δ*abh* mutant strains (Figures. 2.3 and S2.1). Indeed, even the Δ*sigX* single mutant greatly reduced *sunAT* mRNA levels,

suggesting that σ^X is the major ECF σ factor regulating *sunAT* expression. Consistent with the results of spot-on-lawn assay (Figure 2.2A), the *sunAT* mRNA levels in the Δ*abrB* mutant were not significantly different from that in WT. This indicates that AbrB repression is effectively overcome under these growth conditions. This is expected for growth on plates since the producer cells are at high density and AbrB repression is relieved upon entry into stationary phase. It is surprising that AbrB repression was not apparent during logarithmic growth when the RNA was isolated for slot blot analysis (Figure 2.3). Since Δ*abh* failed to produce sublancin, whereas the double deletion of Δ*abh abrB* displayed high-level expression, we suggest that AbrB represses *sunA* transcription in the Δ*abh* mutant even though this effect was not obvious in WT. One possible explanation is that *abrB* expression itself may be derepressed in an *abh* mutant. These two paralogs are known to bind to related sequences and it has previously been suggested that AbrB regulates *abh* transcription (32). Our results suggest that the converse may also be true. Taken together, these results are consistent with the hypothesis that Abh functions as an antagonist of AbrB-mediated repression in order to allow sublancin synthesis.

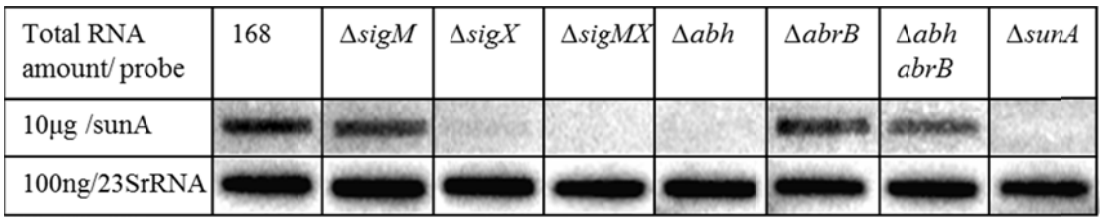


Figure 2.3. σ^X is the major σ factor regulating *sunAT* transcription, and Abh counteracts the repression of AbrB at *sunAT* transcription. The level of *sunAT* transcription in 168 and its derivative mutant strains were detected by RNA slot blot analysis using ^{32}P-labelled *sunA* DNA probe. 23S rRNA was used as a loading control.

σ^X and σ^M dependent transcription of Abh activates *sunAT* transcription. Previous studies have shown that σ^X and σ^M can both contribute to the expression of *abh* (11, 18) and an ECF σ type consensus sequence is found upstream of *abh* (see Figure 2.5A). Our genetic analyses also suggested that the primary effects of σ^X and/or σ^M in activating sublancin synthesis is dependent on Abh which then indirectly activates *sunA.* In order to test this model, a xylose-inducible *abh* allele was introduced into the *lacA* locus in the background of 168, Δ*sigM,* Δ*sigX* and Δ*sigMX* strains (strains HB10123, HB10124, HB10128 and HB10137, respectively). Induction of *abh* expression restored sublancin production to each of these single and double mutants (Figure 2.4), effectively bypassing the requirement for ECF σ factors. Note that the sizes of the inhibition zones in this assay (Figure 2.4) are somewhat reduced relative to that seen in comparable assays (Figure 2.2A, upper panel). This might be due to the presence of xylose in this medium (to induce *abh* transcription), as it has been reported that xylose can modify sublancin and interfere with its bacteriocin activity (9). Nevertheless, it is clear that ectopic induction of Abh restores sublancin production in the *ΔsigMX* strain.

The regulation of *abh* transcription by σ^X and σ^M was further confirmed using β-galactosidase assays with a transcriptional reporter fusion (P_{abh}-*lacZ*). The P_{abh}-*lacZ* construct was introduced at *amyE* in the 168, single, and double mutants of *sigM* and *sigX* (strains HB10147, HB10148, HB10149 and HB10150). P_{abh} activity was slightly reduced in the Δ*sigM* mutant, and strongly reduced in the Δ*sigX* mutant (Figure 2.5B). No β-galactosidase activity was detected in the Δ*sigMX* double mutant.

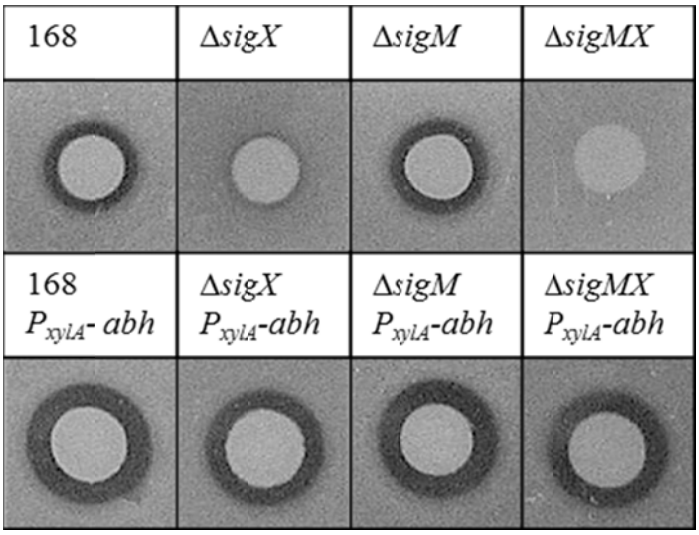


Figure 2.4. Ectopic Abh expression bypasses the requirement for σ^X and σ^M in sublancin production. 168 and its derivative mutants were spotted on a lawn of CU1065. The induction of Abh expression with xylose restores sublancin production in the strain harboring a double deletion of the *sigX* and *sigM* genes (Δ*sigMX*).

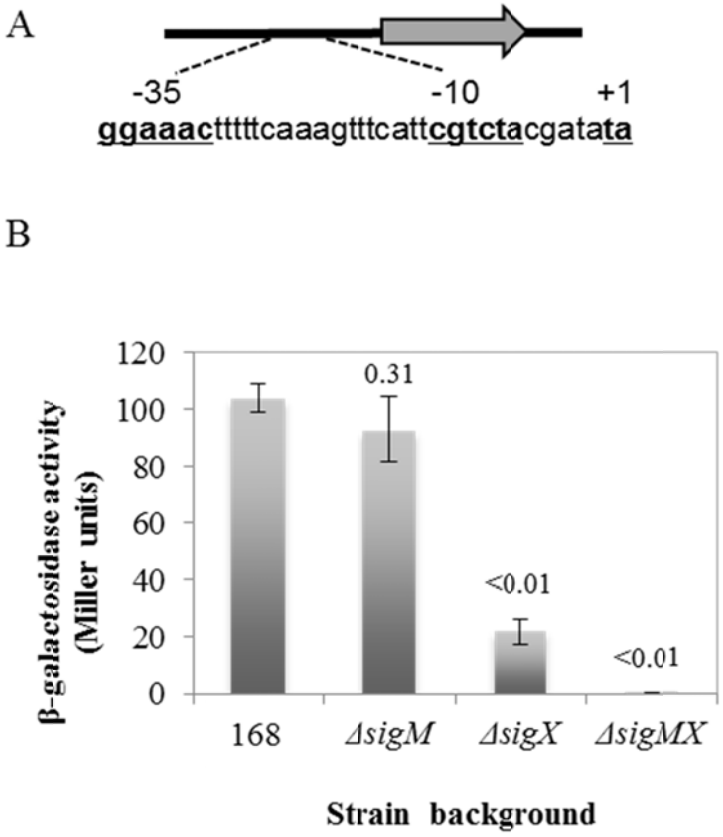


Figure 2.5. *abh* expression is regulated by σ^X and σ^M. (A) Schematic map of the *abh* gene and its upstream region. The promoter region recognized by σ^X and σ^M is underlined in bold and the transcriptional start site indicated by +1. (B) The regulation of *abh* transcription was measured from the *abh* promoter fused to *lacZ*. The β-galatocidase activity of the P_{abh}-*lacZ* fusion reported was measured in strain 168 and its derivative mutant strain backgrounds grown to exponential phase in LB, as indicated on the x-axis. The numbers above each bar are the P values for a paired Student T-test comparing 168 to mutants.

This result suggests that the activity of P_{abh} requires σ^X or σ^M and that σ^X is the major regulator under these growth conditions. These results are entirely consistent with the regulatory effects of σ^X and σ^M as observed by monitoring the *sunAT* transcript levels (Figure 2.3).

Previous studies, using primer extension mapping, had suggested that the *abh* promoter region might contain multiple, closely-spaced start sites, only one of which was dependent on σ^X (18). However, using 5'-RACE analysis of cDNA ends (Figure S2.2) we observed that transcription initiated at the identical adjacent positions, albeit with slightly altered frequencies, in either the *sigX* mutant (in which transcription reflects σ^M activity) or in the *sigM* mutant (indicative of σ^X activity). These results suggest that both of these ECF σ factors recognize the same promoter sequences, consistent with the previously proposed consensus sequences for σ^X and σ^M (21).

Sublancin expression is controlled by a complex regulatory network. Most antibiotics produced by *B. subtilis* are synthesized upon entering stationary phase or induced by stress or quorum sensing (reviewed in detail in (31) and (19)). It has long been known that antibiotic production and resistance are regulated, in a large part, by AbrB. Indeed, nutrient deprivation leads to a gradual increase in the phosphorylation, and hence activity, of Spo0A, the master regulator of sporulation (14). Mutants lacking *spo0A* are pleiotropic and also fail to express antibiotics and associated resistance functions. A *spo0A abrB* mutant strain restores regulation of antibiotic production, but fails to restore the ability to sporulate. It is now appreciated that *abrB* is a high affinity target for the Spo0A~P repressor and that relief of AbrB repression

upon nutrient depletion leads to the expression of antibacterial compounds such as SdpC, a toxic peptide, and the Skf cannibalism factor (12, 13, 27).

In addition to AbrB, the paralogous regulator Abh also plays a complex role in coordinating the synthesis of antibacterial compounds. Previous studies have shown that Abh can act both negatively and positively to affect expression of antibacterial compounds. Like AbrB, Abh acts as a negative regulator at the *skfA* and *sdpA* promoter regions (32). In contrast to AbrB, however, Abh acts positively on expression of sublancin as confirmed here. We have shown that the effect of *abh* on sublancin expression is epistatic to *abrB* and that in an *abrB* mutant strain, the requirement for *abh* is bypassed. Expression of *abh* is itself controlled by numerous transcription factors. As shown here, *abh* transcription requires the activity of either σ^X or σ^M with σ^X as the major regulator under these growth conditions. Expression of *abh* is repressed by AbrB, which also negatively regulates its own synthesis.

Taken together, a complex regulatory circuitry emerges (Figure 2.6) in which nutritional and growth phase dependent signals converge to elevate levels of Spo0A~P leading to an initial repression of *abrB* transcription. In addition, AbrB activity is subject to multiple levels of post-transcriptional control including antagonism by the AbbA antirepressor (1), and antagonism by Abh. The latter is likely to be promoter-specific and may reflect the overlapping DNA-binding specificity of these two paralogous transcription factors. Those cells that are lysogenic for SPβ and expressing either σ^X or σ^M, and therefore expressing Abh, will proceed to express the potent lantibiotic sublancin. σ^X or σ^M also appears to have some modest activation effect on sublancin production which is independent of Abh or AbrB. In addition, AbrB is

known to repress both *sigW* and many target operons for σ^W (28). Among the operons under σ^W control, the *yqeZ-yqfAB* operon provides a background level of resistance to sublancin for those cells lacking SPβ and therefore lacking the cognate immunity protein for sublancin, SunI. YqeZ encodes a putative transmembrane protease and YqfA and YqfB are both putative membrane anchored proteins.

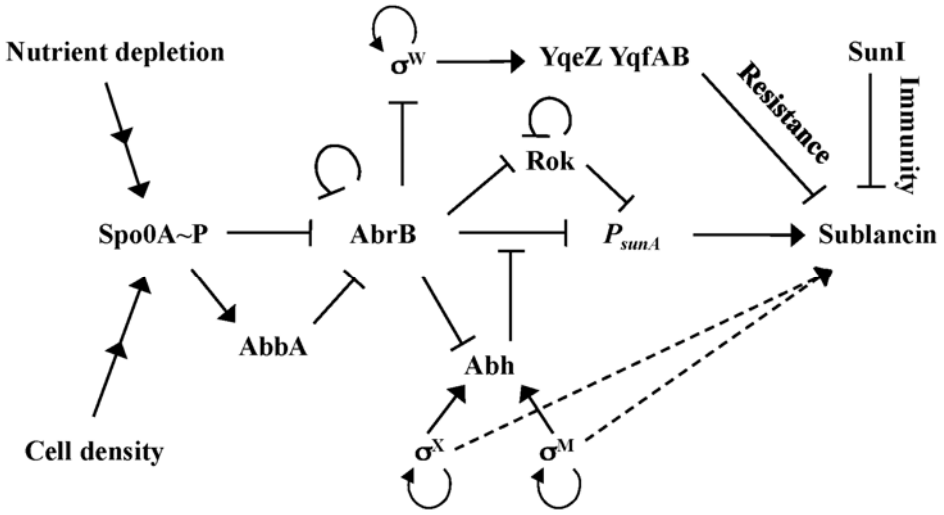


Figure 2.6. Regulatory network of sublancin synthesis and resistance in *B. subtilis*. Positive regulatory effects are indicated by straight arrows, putative positive regulatory effects are indicated by arrows with dash lines, and negative regulations are indicated by "—|", and See text for detailed discussion.

2.4 Concluding remarks

ECF σ factors are known to provide resistance against various cell envelope antibiotics (2, 4, 11, 21). This report establishes that ECF σ factors also regulate antibiotic production in *B. subtilis*. When spotted on lawns of *Bacillus* spp. strains, strain 168 was able to inhibit the growth of various strains and this inhibition was due to sublancin synthesis. A double deletion of σ^X and σ^M eliminated the production of sublancin due to an inability to express Abh, an antagonist of AbrB-mediated repression at the *sunA* promoter. Indeed, both AbrB and Abh have previously been shown to bind to overlapping regions in the *sunA* regulatory region (32), but it remains unclear how AbrB and Abh work together to regulate *sunA* expression. One simple model suggests that Abh binding to this region (either alone or together with AbrB) alters the protein-DNA complex such that the promoter is now available for interaction with RNA polymerase. Clearly, the production of antibiotic and their associated resistance determinants, as here exemplified by sublancin, is subject to an enormously complicated control network. An ultimate, but perhaps still distant, goal will be to model these complex interactions to develop a predictive model of this regulatory circuitry.

2.5 Acknowledgements.

We would like to thank Yoshito Sadaie (Saitama University, Japan) for strain 168, Mark Strauch (University of Maryland) for strain SWV121.

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The supplementary information can be found at the end of this chapter.

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2.6 Supplementary Information

Table S2.1. List of lawn strains tested for susceptibility to compounds produced by strains of 168 and ΔMWX.

Strains showing less susceptibility to ΔMWX strain than strain 168	**Strains showing similar susceptibility to strain 168 and ΔMWX strain**
B.subtilis subsp spizizenii (2A8) *	*B.sphaerius WT isolate 1593 (13A1)*
B.subtilis subsp spizizenii (2A9)	*B.thuringiensis subsp. Thuringiensis NRRL-B403 (4A1)*
B.licheniformis ATCC14580 (5A36)	*B.thruingiensis subsp. thochigiensis HD868 (4Y1)*
B.atrophaeus NRS-213 (11A2)	*B.licheniformis 749 (5A20)*
B.atrophaeus ESM (12A1)	*B.cereus ATCC14579 (6A5)*
B.amyloliquefaciens FZB42 (10A6)	*B.sphaerius ATCC14577 (13A6)*
B.amyloliquefaciens FZB42, sfp-	*B.sphaerius NRS400 (13A9)*
B.pumilus ATCC7061 (8A3)	
B.licheniformis ATCC8480 (5A1)	
B.megaterium strain 899 (7A1)	
B.megaterium ATCC19213 (7A2)	
B.megaterium ATCC14581 (7A36)	
B.megaterium QMB1551 (7A16)	
B.pumilus BP1 (8A1)	
B.amyloliquefaciens NRL B-14393 (10A5)	
B.atrophaeus WT isolate SB512 (11A1)	

* The *Bacillus* Gene Stock Center Accession number of each strain is shown in parentheses.

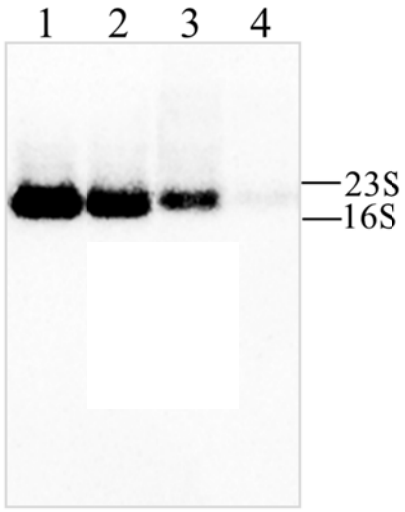


Figure S2.1. *sunA* and *sunT* are co-transcribed. Ten micrograms of total RNA from four strains (1, 168; 2, Δ*sigM*; 3, Δ*sigX*; 4, Δ*sigMX*) were subjected to Northern blot analysis using ^{32}P-labeled *sunA* DNA as probe. 23S RNA (3kb) and 16S RNA (1.5kb) were used as RNA size indications.

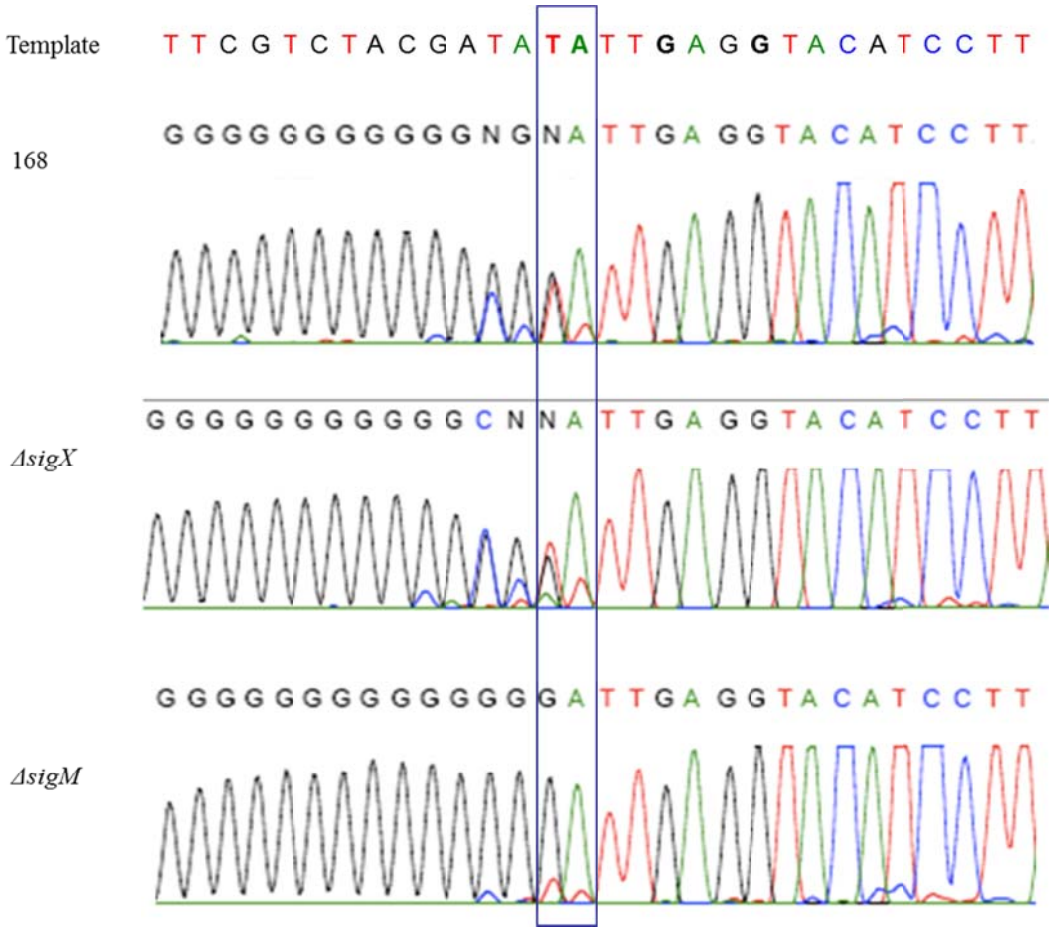


Figure S2.2. σ^X and σ^M initiate from the same transcriptional start site of *abh*. The *abh* transcriptional start sites of 168, Δ*sigX,* and Δ*sigM* were mapped using 5'-RACE, and are highlighted in the box.

CHAPTER 3

TRANSCRIPTOMIC AND PHENOTYPIC CHARACTERIZATION OF A *BACILLUS SUBTILIS* STRAIN WITHOUT EXTRACYTOPLASMIC FUNCTION SIGMA FACTORS

Bacillus subtilis encodes seven extracytoplasmic function (ECF) σ factors. Three (σ^M, σ^W and σ^X) mediate responses to cell envelope active antibiotics. The functions of σ^V, σ^Y, σ^Z, and σ^{YlaC} remain largely unknown, and strong inducers of these σ factors and their regulons have yet to be defined. Here, we define transcriptomic and phenotypic differences under non-stress conditions between strains carrying deletions in all seven ECF σ factor genes (Δ7ECF), a ΔMWX triple mutant, and the parental 168 strain. Our results identify >80 genes as at least partially dependent on ECF σ factors and, as expected, most of these are dependent on σ^M, σ^W or σ^X which are active at a significant basal level during growth. Several genes, including the *eps* operon encoding enzymes for exopolysaccharide (EPS) production, were decreased in expression in Δ7ECF but affected less in ΔMWX. Consistent with this observation, Δ7ECF (but not ΔMWX) showed reduced biofilm formation. Extending previous observations, we also note that ΔMWX is sensitive to a variety of antibiotics and Δ7ECF is either as sensitive as, or slightly more sensitive than, the ΔMWX strain to these stressors. These findings emphasize the overlapping nature of the seven ECF σ factor regulons in *B. subtilis*, confirm that three of these (σ^M, σ^W or σ^X) play the

dominant role in conferring intrinsic resistance to antibiotics, and provide initial insights into the roles of the remaining ECF σ factors.

The results of this study were published in Luo Y, Asai K, Sadaie Y, Helmann JD. Journal of Bacteriology. 2010 Nov;192(21):5736-45.

3.1 Introduction

Bacillus subtilis harbors seven extracytoplasmic function (ECF) σ factors (σ^M, σ^W, σ^X, σ^Y, σ^Z, σ^V, and σ^{YlaC}) that collectively control a variety of functions related to cell envelope homeostasis and defenses against cell envelope-active compounds (27). The physiological roles of three ECF σ factors (σ^M, σ^W, and σ^X) have been previously examined, their target regulons identified, and phenotypes associated with their inactivation documented. Physiological functions attributable to the remaining 4 ECF σ factors (σ^V, σ^Y, σ^Z, and σ^{YlaC}) are still largely unknown.

To date, most identified ECF σ factor target genes are dependent on one or more of σ^M, σ^W, or σ^X. Since these three σ factors are all active, at least at a low level, during growth and their regulons overlap, we refer to these three as the MWX group. The σ^W regulon is activated by various cell wall antibiotics, alkali shock, and other stresses affecting the cell envelope (14, 42, 49). The σ^W regulon includes ~30 operons (~60 genes), many of which mediate intrinsic resistance to antimicrobial compounds. For example, the σ^W-dependent *fosB* gene provides resistance to the cell wall-acting antibiotic fosfomycin (11) and the *yqeZ yqfAB* operon mediates protection against the antibiotic peptide sublancin (10). σ^X controls several genes involved in modification

of the overall charge of the cell envelope including the *dlt* and *pssA* operons (12, 29). Deletion of *sigX* results in higher susceptibility to cationic antimicrobial peptides. σ^M is induced by a number of stresses including high salt, heat, ethanol, acid, phosphate starvation, superoxide stress, and the cell wall-acting antibiotics bacitracin, vancomycin, and cationic antimicrobial peptides (17, 28, 36, 42, 45). A *sigM* mutant has higher susceptibility to bacitracin, paraquat and high salinity (13, 15, 36, 45). Genes controlled by σ^M are important for cell wall biosynthesis and modification, shape determination and cell division, and detoxification, suggesting a role for σ^M in maintaining cell envelope integrity in diverse environments (21).

There is significant overlap in recognition of target promoters amongst σ^M, σ^W, and σ^X and this complicates attempts to clearly delineate their individual regulons. For example, the bacitracin resistance gene *bcrC* is under the dual control by σ^M (its primary activator) and σ^X (13, 36). σ^M and σ^X both can activate transcription of the *dlt* operon, although σ^X is the primary activator (12, 21). The regulatory redundancy among ECF σ factors in *B. subtilis* often masks the effect of single ECF σ factor deletions. Indeed, a triple ΔMWX deletion mutant displays additional phenotypes not found amongst the three individual deletion mutants. These include higher susceptibility to several detergents and to the cell wall-acting antibiotics polymyxin B, D-cycloserine, and ampicillin (35). Collectively, those genes dependent on σ^M, σ^W, and/or σ^X define the σ^{MWX} regulon. Our current understanding regarding the physiological importance of the remaining four ECF σ factors (σ^V, σ^Y, σ^Z, σ^{YlaC}) is very limited. It seems plausible that the seemingly cryptic nature of these regulators

may be due to the lack of well-defined inducing conditions, overlapping promoter recognition with other ECF σ factors, or both. We refer to these as the VYZ-YlaC group.

As one approach to define the roles of each of the 7 ECF σ factors, transcriptome analysis was previously conducted in strains after 2 hrs of induction of each σ factor and large and often overlapping sets of genes were identified as possible targets (3). In a separate study, induction of σ^V revealed a regulon comprised largely of previously identified members of the σ^{MWX} regulon (52). This overlap might explain the failure to associate significant phenotypes with null mutants of *sigV*. In contrast, σ^Y was shown to directly activate transcription from only two target promoters: the one that controls the *sigY* operon (*sigY-yxlCDEFG*) and one for *ybgB* (16). The functions of σ^{YlaC} (37) and σ^Z are presently unclear and their cognate regulons are not well defined.

Here, we have exploited a recently described *B. subtilis* strain lacking ECF σ factors (Δ7ECF) (2) to identify unique transcriptional and phenotypic signatures associated with the four seemingly cryptic ECF σ factors. Altogether, we identified >80 genes as at least partially ECF σ factor dependent in the absence of artificial σ factor induction. A small subset are expressed at a much lower level in Δ7ECF when compared to ΔMWX, implying that these may be either direct or indirect targets for σ^V, σ^Y, σ^Z, or σ^{YlaC}. These genes include the *eps* operon, which encodes genes required for exopolysaccharide (EPS) production implicated in biofilm formation.

3.2 Materials and Methods

Bacterial strains and growth conditions. Strains used in this study are listed in Table 3.1. Bacteria were grown in Luria-Bertani (LB) broth at 37°C with vigorous shaking or on solid LB medium containing 1.5% Bacto agar (Difco). The following antibiotics were used for selection when necessary: spectinomycin 100 μg/mL, kanamycin 10 μg/mL, chloramphenicol 10 μg/mL, tetracycline 5 μg/mL, and erythromycin 1 μg/mL with lincomycin 25 μg/mL (mls: macrolide-lincomycin-streptogramin B resistance).

Gene deletions were generated by replacing genes with antibiotic resistance cassettes using long-flanking-homology PCR as described (36, 48). Chromosomal DNA transformation was performed as described (26). The Δ7ECF strain contains unmarked deletions of all seven ECF σ factor-encoding genes as previously described (2).

RNA preparation and microarray analyses. Cultures of stains 168, ΔMWX, and Δ7ECF were grown to mid-log phase (OD_{600} of 0.4). Total RNA was isolated from three different biological replicates with the RNeasy Mini Kit (Qiagen Sciences, Maryland). After DNase treatment with TURBO DNA-free™ (Ambion), RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop Tech. Inc., Wilmington, DE). cDNA labeling and microarray analysis were performed as described (24). Three microarrays with biological triplicates were performed for each microarray comparison. Images were processed and normalized using the GenePix Pro 4.0 software package which produces (R, G) fluorescence intensity pairs for each gene. Each expression value is represented by at least six separate

Table 3.1. *B.subtilis* strains used in this study.

Strains	Genotype	Construction or reference
168	*trpC2*	Lab strain
CU1065	*trpC2 attSP*β	Lab strain
JH642	*trpC2 pheA1*	(5)
NCIB3610	undomesticated wild strain	Lab strain
HB10107	168 *sigM::tet sigX::kan sigW::mls*	(34)
BSU2007	168 Δ*sigM* Δ*sigX* Δ*sigW* Δ*sigY* Δ*sigV* Δ*sigZ* Δ*ylaC*	(2)
HB0009	CU1065, *sigY*::*mls*	(16)
HB0911	CU1065 *sigV*::*cat*	(35)
HB0915	CU1065 *ylaC::spec*	(35)
HB0032	CU1065 *sigZ*::*kan*	(15)
HB10101	168 *sigV::cat*	ChrDNA HB0911→168
HB10108	168 *sigY::mls*	ChrDNA HB0009 →168
HB10109	168 *sigZ::kan*	ChrDNA HB0032 →168
HB10233	168 *ylaC::spec*	ChrDNA HB0915→168
HB10234	168 *sigY::mls ylaC::spec*	ChrDNA HB10108→HB10233
HB10235	168 *sigZ::kan sigV::cat*	ChrDNA HB10109→HB10101
HB10236	168 *sigV::cat sigY::mls sigZ::kan ylaC::spec*	ChrDNA HB10234→HB10235
YC125	NCIB3610 Δ*epsH*::*tet amyE*::P_{lutA}-*lacZ* (*cat*)	(18)
HB10223	168 *epsH::tet*	ChrDNA YC125 →168
HB10158	168 *amyE::*P_{spac}*-abh* (*cat*)	ChrDNA BZH73→168
HB10224	168 Δ*sigM* Δ*sigX* Δ*sigW* Δ*sigY* Δ*sigV* Δ*sigZ* Δ*ylaC amyE::*P_{spac}*-abh* (*cat*)	ChrDNA HB10158→ BSU2007
HB10225	168 *sigM::tet sigX::kan sigW::mls amyE::*P_{spac}*-abh* (*cat*)	ChrDNA HB10158→ HB10107
JMA208	JH642 *immR*::*cat*	(5)
JMA222	JH642 ICE*Bs1*^{0}/cured of ICE*Bs1*	(5)
BZH73	JH642 *abh::kan amyE::*P_{spac}*-abh* (*cat*) *thrC::* P_{sunA}*-lacZ* (*spec*)	(44)

measurements (duplicate spots on each of three arrays). Mean values and standard deviations were calculated with MS Excel. The normalized microarray datasets were filtered to remove those genes that were not expressed at levels significantly above background in either condition (sum of mean fluorescence intensity <20). In addition, the mean and standard deviation of the fluorescence intensities were computed for each gene and those where the standard deviation was greater than the mean value were ignored. The fold induction values were calculated using the average signal intensities from the three arrays with strains within microarray experiment pairs.

The complete set of raw and normalized data for each of the triplicate DNA microarray experiments involving strains *B. subtilis* 168, ΔMWX and Δ7ECF is available at the Gene Expression Omnibus database (http://ncbi.nlm.nih.gov/geo/) under accession no. GSE22930.

Hierarchical Clustering analysis. Genes with at least 3 fold change in at least one microarray comparison pairs were subjected to hierarchical clustering analysis with Gene Cluster 3.0 software (22). The $\log_2$ ratios of fold changes were used, and the resulting cluster was visualized with Treeview 1.60 (22).

Phenotype microArray and disk diffusion. Phenotype MicroArray™ (PM) assays were performed by Biolog (Biolog Inc. CA.). The Biolog plates used for these analyses were PM1~PM20 as described on the Biolog web site (http://www.biolog.com/PM_Maps.html). Cell growth was monitored by measuring the respiration-dependent color change of Biolog Redox Dye D in each well. Incubation and recording of phenotypic data was performed in the Omnilog™ -Station. A time course for dye color formation (respiration) for cells was generated and the

difference in growth rates between different strains was calculated using the Omnilog software. The growth differences were reported as arbitrary units. Positive values indicate that the mutant showed greater rates of growth (or respiration) than the control wild type strain. The negative value indicates the mutant showed lower growth (or respiration) rate than the control wild type strain. The PM assay was performed twice. The average values of these two independent experiments were reported and used for further analysis. The significant hits (as defined by Biolog) are listed in Table S3.1 (supplementary information) for the ΔMWX *versus* 168, and Δ7ECF *versus* 168 comparisons. The original PM data is available upon request.

Disk diffusion was performed as described (35). Briefly, strains of 168, ΔMWX and Δ7ECF were grown to mid-log phase (OD_{600} of 0.4) in LB medium at 37°C with aeration. A 100 µl aliquot of these cultures was mixed with 4 ml of 0.7% LB soft agar (kept at 50°C) and directly poured onto LB plates (containing 15 ml of 1.5% LB agar). The plates were then dried for 20 min in a laminar airflow hood. Filter paper disks containing the chemicals to be tested were then placed on the top of the agar and the plates were incubated at 37°C overnight. The next day, the diameters of the inhibition zones (clearance) were measured. The zones of inhibition were reported after subtraction of the diameter of the filter paper disk (6.5 mm). The following chemicals and quantities were used in the disk diffusion assays: dodecyltrimethyl ammonium bromide (DTAB) 250 µg, triton X-100 5µl of 10% solution, amitriptyline 250 µg, polymyxin B 50 µg, bacitracin zinc 250 µg, fosfomycin 500 µg, ampicillin 10 µg, penicillin G 10 unit, aztreonam 30 µg, cefuroxime 30 µg, ciprofloxacin 50 µg, ofloxacin 10 µg, zinc chloride 500 mM, and polymyxin B 50 µg.

Biofilm formation microtiter plate assay. Biofilm formation assays were performed as described (25, 33) with modifications. *B.subtilis* strains were grown in Biofilm Growth (BG) medium, which is LB broth supplemented with 100 mM MOPS (pH 7.0), 1 mM $MgSO_4$ and 0.1% glucose. IPTG (1 mM, final concentration) was added when required. Exponentially growing cultures (OD_{600}, 0.4) were diluted to an OD_{600} of 0.01 with BG medium, and 100 µl aliquots of the freshly inoculated medium were dispensed into wells of a 96-well polyvinyl chloride microtiter plate (Falcon 353911 flexible U-bottom plates, Becton-Dickinson Labware). Cells were statically incubated at 37°C for 65 hours. Cells were aerated by pipetting up and down twice every 12 hours; the BG medium with planktonic cells was replaced with fresh BG media every 24 hours. After 65h incubation, the unbound planktonic cells were removed by gentle aspiration. The wells with adherent biofilm cells were gently rinsed twice with 100 µl washing buffer (100 mM MOPS, 1 mM $MgSO_4$, pH7.0), followed by heat fixation at 70°C for 10 min, and stained with 200 µl per well of 1% crystal violet (dissolved in washing buffer) for 20 min. The wells were rinsed under running deionized water after staining. The bound cystal violet stains were solubilized with 200 µl of ethanol-acetone (4:1, vol./vol.) for 20 min. The solutions were transferred to a fresh 96 well polystyrene plate, and absorbance measured at OD_{570} using a Tecan Rainbow microplate reader. Biological triplicates of each strain were assayed in 21 wells in the same plate, and the biofilm assays were performed at least three times. The data from the "same day" experiments were reported.

3.3 Results and Discussion

Identification of genes regulated by ECF σ factors using cDNA microarray analysis. Previous microarray analyses have been reported for either some single mutant strains or over-expression of single σ factors (*sigW, sigX, sigM,* and *sigY*) (12, 14, 16, 21), but these studies likely missed some targets that can be transcribed by more than one ECF σ factor. Other studies have investigated transcriptome changes after prolonged (2 hrs) artificial σ factor induction, but the significance of these preliminary findings has not been further investigated (3). Whereas regulon overlap has emerged as a significant confounding factor in assigning ECF σ regulons in *B. subtilis* (35), there is no evidence to date of σ cascades in which one ECF σ acts indirectly by activating transcription of another in *B. subtilis*. Six of seven ECF σ factor (except σ^Z) are encoded in operons that are thought to be largely autoregulated under inducing conditions (51). It is within this conceptual framework that we have sought to define the roles of ECF σ factors.

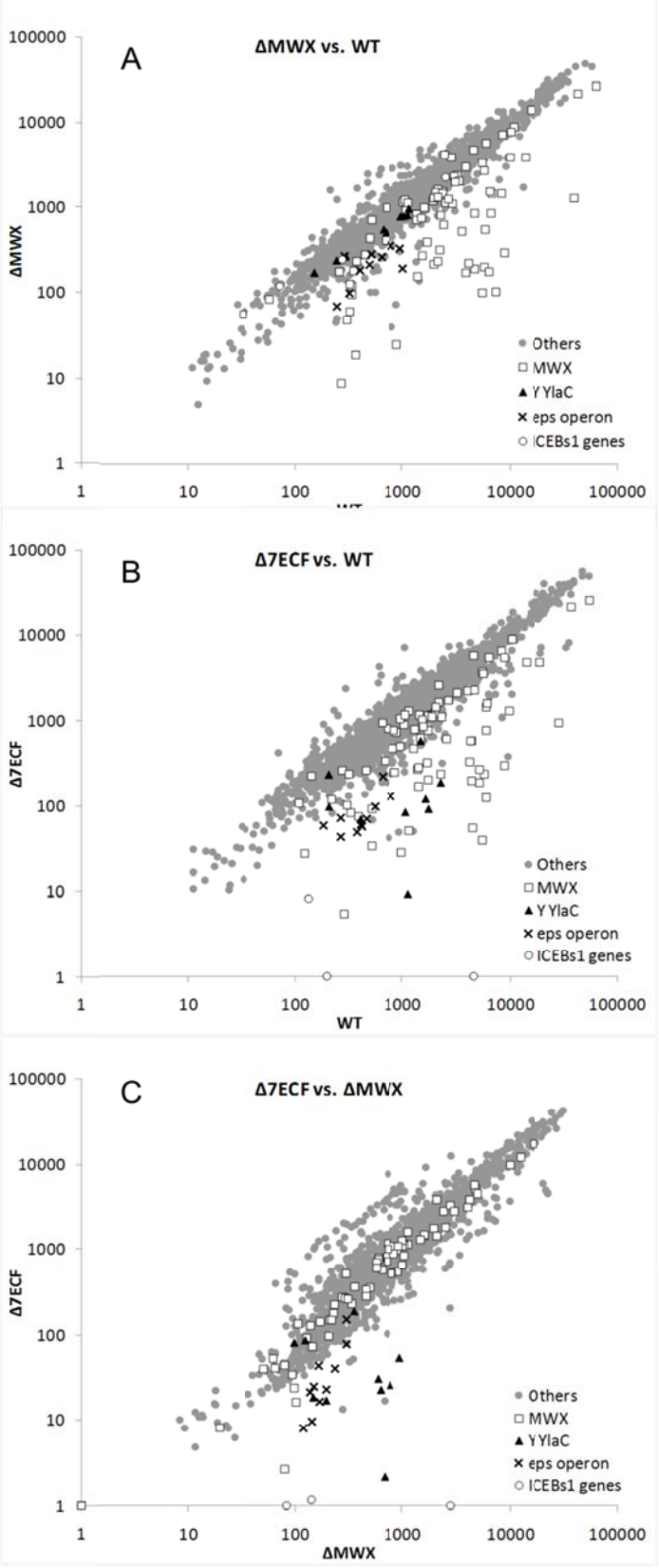
Here, we set out to identify genes that are at least partially dependent on one or more ECF σ factors during growth and, further, to identify those genes or functions that might be dependent on the less studied σ factors: σ^V, σ^Y, σ^Z, and σ^{YlaC} (the VYZ-YlaC group). We carried out cDNA microarray analyses for logarithmically growing cells (LB medium) with strains 168 (wild type, WT), a strain with null mutations in three ECF σ factor genes (*sigM*, *sigW,* and *sigX*; ΔMWX), and a mutant strain harboring null mutations of all 7 ECF σ factors (Δ7ECF). Genes that are dependent only on σ^M, σ^W and/or σ^X (the σ^{MWX} regulon) should be expressed at a lower level in

both ΔMWX and Δ7ECF when compared to WT, but should remain unchanged in the comparison of Δ7ECF *versus* ΔMWX. Genes that are largely dependent on σ^V, σ^Y, σ^Z, and/or σ^{YlaC} should be expressed at a lower level in Δ7ECF *versus* WT, and Δ7ECF *versus* ΔMWX, but remain relatively unchanged in the ΔMWX *versus* WT comparison.

As expected, the expression of many of the known σ^{MWX} regulon members was reduced in strain ΔMWX relative to WT, whereas the known autoregulated operons encoding σ^Y and σ^{YlaC} remained unchanged (Figure 3.1A). In the comparison between Δ7ECF and WT, the known regulons of σ^M, σ^W, σ^X, σ^Y and σ^{YlaC} were reduced in expression, as expected (Figure 3.1B). In the comparison between strains Δ7ECF and ΔMWX (Figure 3.1C), a majority of σ^{MWX} dependent genes remained unchanged. This indicates that, under these growth conditions, the other four ECF σ factors (σ^V, σ^Y, σ^Z, and σ^{YlaC}) contribute little to the expression of the σ^{MWX} regulon. These transcription patterns match well with expectations based upon previously published reports regarding ECF σ regulon composition (35).

Some known σ^{MWX} regulon genes did not display differential expression in the comparisons between ΔMWX *versus* WT, and Δ7ECF *versus* WT (Figure 3.1A and 3.1B). This likely reflects the fact that these genes were previously assigned as ECF σ-dependent under inducing conditions, such as treatment with antibiotics, and they may not be significantly dependent on ECF σ factors under the non-stress conditions of the current study (due, for example, to the presence of promoters recognized by non-ECF σ factors). Presumably, only genes that are strongly dependent on ECF σ

Figure 3.1. Scatter plot of cDNA microarray analyses. The average signal intensities of each microarray experiments were plotted. The known regulons of σ^M σ^W σ^X, σ^Y σ^{YlaC}, genes of *eps* operon, and ICE*Bs1* elements are differentially labeled.


A
ΔMWX vs. WT
ΔMWX
100000
10000
1000
100
10
1
1
10
100
1000
10000
100000
Others
MWX
Y YlaC
eps operon
ICEBs1 genes
B
Δ7ECF vs. WT
Δ7ECF
WT
C
Δ7ECF vs. ΔMWX
Δ7ECF
ΔMWX

factors for their basal expression during log phase will show significant differences in our analysis.

In order to further delineate the regulons of these ECF σ factors, we performed a hierarchical clustering analysis with genes displaying significant differences in expression (>3 fold changes) in at least one of the three microarray comparisons (Figure 3.2). Clustering is an un-supervised method, with no assignment of regulon information before the analysis. Since ECF σ factors are positive regulators, we focused on genes that were reduced in expression in the absence of ECF σ factors. Based on the clustering analysis and expression pattern, we further classified genes into three categories: target genes for σ^{MWX}, targets of σ^{Y} and σ^{YlaC}, and additional targets significantly reduced in Δ7ECF compared to ΔMXW (Table 3.2). The latter group of genes presumably includes genes dependent on one or more of the poorly characterized VYZ -YlaC group of ECF σ factors, either alone or together with MWX. Under these growth conditions we did not detect signals in the microarray for either *sigV* or *sigZ*. This suggests that these σ factors are expressed poorly if at all and therefore their contribution to gene expression is likely to be minimal. When genes within an operon were located in a different section of the cluster, the average fold change for the operon was used for assigning the apparent regulon.

The σ^{MWX} target genes. Thirty two genes were classified as putative σ^{MWX} target genes since their expression was significantly reduced in both the ΔMWX and Δ7ECF strains when compared to WT, while there was little difference in the ΔMWX *versus* Δ7ECF comparison. The majority of these genes were previously identified as σ^{MWX}

regulon members, thereby validating our experimental approach. This analysis did identify three new candidate σ^{MWX} target genes: *yxiT*, *yhjA* and *des*.

Both *yxiT* and *yhjA* are unknown function genes that encode small peptides. Typical σ^{MWX} promoter sequences were not found upstream of either gene, suggesting that their regulation by σ^{MWX} is likely to be indirect. The *des* gene, encoding a delta5 lipid desaturase (1), is induced by the DesKR two component system in response to conditions that reduce membrane-fluidity (19, 20). No candidate σ^{MWX}-type promoters were found within 1kb upstream of either *des* or *desKR*, suggesting that reduced expression in the *sigMWX* mutant may be an indirect consequence of altered membrane homeostasis. For example, the σ^{MWX} regulon is known to include enzymes for phospholipid synthesis (*pssA* and *psd* (12)), lipoteichoic acid synthesis (*yfnI* (21, 30)), and D-alanylation of teichoic acids (the *dltABCDE* operon (12, 21)). Whether altered transcription of some or all of these genes contributes to the observed *des* induction is presently unknown.

The σ^{Y} and σ^{YlaC} target genes. We detected 36 genes that closely cluster with the autoregulated *sigY* operon and the *ylaC* gene. Based on this clustering, and the apparent lack of σ^{V} and σ^{Z} expression under these conditions, these genes were classified as putative σ^{Y} and σ^{YlaC} target genes. These genes showed less than 2-fold change when comparing their expression in strain ΔMWX and WT, but more than 3-fold change in the comparisons between the Δ7ECF and WT strains, or the Δ7ECF and ΔMWX strains, or both (Table 2). Previously published information regarding σ^{Y}

Figure 3.2. Hierarchical clustering analysis of genes that are down regulated at least three fold in at least one of the three microarray analyses. Green indicates down regulation, and red indicates up regulation. The known ECF σ regulon are indicated by the * M,W,X,Y,YlaC followed the gene names.

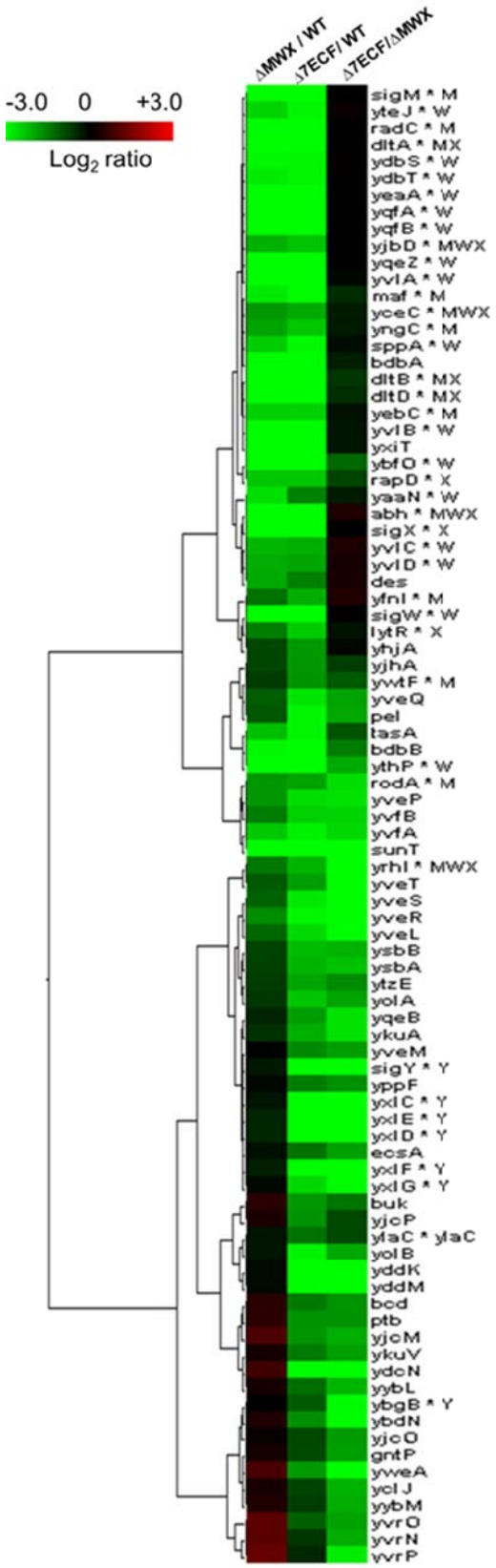

ΔMWX / WT
ΔTECF/ WT
ΔTECF/ΔMWX
-3.0 0 +3.0
Log2 ratio
sigM * M
yteJ * W
radC * M
dltA * MX
ydbS * W
ydbT * W
yeaA * W
yqfA * W
yqfB * W
yjbD * MWX
yqeZ * W
yvlA * W
maf * M
yceC * MWX
yngC * M
sppA * W
bdbA
dltB * MX
dltD * MX
yebC * M
yvlB * W
yxiT
ybfO * W
rapD * X
yaaN * W
abh * MWX
sigX * X
yvlC * W
yvlD * W
des
yfnI * M
sigW * W
lytR * X
ythjA
yjhA
ywtF * M
yveQ
pel
tasA
bdbB
ythP * W
rodA * M
yveP
yvfB
yvfA
sunT
yrhI * MWX
yveT
yveS
yveR
yveL
ysbB
ysbA
ytzE
yolA
yqeB
ykuA
yveM
sigY * Y
yppF
yxlC * Y
yxlE * Y
yxlD * Y
ecsA
yxlF * Y
yxlG * Y
buk
yjcP
ylaC * ylaC
yolB
yddK
yddM
bcd
ptb
yjcM
ykuV
ydcN
yybL
ybgB * Y
ybdN
yjcO
gntP
yweA
yclJ
yybM
yvrO
yvrN
yvrP

Table 3.2. Genes that are reduced at least 3 fold in at least one of the three microarray comparisons: ΔMWX *vs.* WT, Δ7ECF *vs.* WT, and ΔMWX *vs.* 7ECF.

Gene /Operon[a]	Fold changes			Regulator[b]	Functions[c]
	ΔMWX/ WT	Δ7ECF/ WT	Δ7ECF /ΔMWX		
σ^{MWX} target genes					
sigM	0.03	0.02	1.0	M	ECF σ factor M
sigX	0.03	0.04	1.0	X	ECF σ factor X
sigW	0.04	0.01	1.0	W	ECF σ factor W
abh	0.02	0.03	1.3	MWX	Transcriptional regulator
yjbD (spx)	0.2	0.2	1.0	MWX	Redox-sensitive regulator enzyme; anti-alpha, global transcriptional regulator
*dlt**ABCDE***	0.03	0.03	1.0	MX	*dltABCDE* operon; D-alanylation of teichoic acids
maf	0.1	0.1	0.7	M	Putative septum formation DNA-binding protein
radC (ysxA)	0.1	0.1	1.0	M	Putative DNA repair protein
rapD	0.2	0.2	0.6	M	Response regulator aspartate phosphatase
yebC	0.2	0.2	0.9	M	Putative integral inner membrane protein
yngC	0.3	0.2	0.8	M	Putative integral inner membrane protein
yceC	0.3	0.2	0.8	M	Putative stress adaptation protein
yfnI	0.4	0.2	1.3	M	Similar to lipoteichoic acid synthase
yeaA	0.01	0.01	1.0	W	Unknown
yqeZ yqfAB	0.03	0.03	1.0	W	Sublancin resistance
ydbST	0.1	0.1	1.0	W	Unknown
yvlABCD	0.1	0.2	1.0	W	Unknown
sppA	0.2	0.1	0.9	W	Signal peptide peptidase
yteJ	0.2	0.1	1.1	W	Putative integral inner membrane protein
yaaN	0.2	0.3	0.8	W	Unknown
lytR	0.4	0.2	0.9	X	Membrane-bound transcriptional regulator
yxiT	0.1	0.1	0.8		Unknown
des	0.2	0.4	1.2	DesKR	Fatty acid desaturase
yhjA	0.6	0.3	0.9		Unknown
σ^{ECF} target genes					
yrhI (bscR)	0.4	0.2	0.03	MX	Transcriptional regulator of *cypB*
rodA	0.3	0.3	0.2	M	Cell-division membrane protein
ywtF	0.6	0.3	0.5	M	Transcriptional regulator; LytR family
ythP	0.1	0.03	0.2	W	Putative ABC transporter
ybfO	0.1	0.1	0.4	W	Putative exported hydrolase

Table 3.2 (Continued)

Gene /Operon[a]	Fold changes			Regulator[b]	Functions[c]
	ΔMWX/ WT	Δ7ECF/ WT	Δ7ECF /ΔMWX		
*sunA-sun**T**-**bdbA**-yolJ-**bdbB***	0.1	0.06	0.3	Abh	Sublancin 168 prduction, modification and transportation
*epsA**BC**DE**FG** **HKJK**LMNO*	0.4	0.2	0.2	SinR/ AbrB	Exopolysaccharide biosynthesis.
tasA	0.2	0.1	0.5	SinR	Major protein component of biofilm matrix
pel	0.5	0.1	0.3	TnrA	Pectate lyase
yjhA	0.6	0.3	0.6		Similar to putative lipoprotein
σ^Y YlaC target genes					
sigY	0.8	0.008	0.003	Y	ECF σ factor Y
*yxl**CDEFG***	0.8	0.1	0.07	Y	*sigY yxlCDEFG* operon; negatively regulator of σ^Y
ylaC	0.8	0.4	0.5	YlaC	ECF σ factor YlaC
ybgB	1.0	0.5	0.1	Y	Unknown
yddK	0.9	0.0002	0.0004		ICE*Bs1* gene
yddM	0.9	0.005	0.008		ICE*Bs1* gene; putative helicase
ydcN (immR)	1.7	0.1	0.01		ICE*Bs1* gene; transcriptional regulator (Xre family)
ybdN	1.3	0.3	0.03		Homologous to antimicrobial protein YbdN in *Bacillus licheniformis*
yolA	0.6	0.2	0.3		spβ prophage genes; secreted
yolB	0.8	0.1	0.3		spβ prophage gene
*ysb**AB***	0.6	0.2	0.2		Homologous to *lrgAB* in *Staphylococcus aureus*
ykuA (pbpH)	0.7	0.2	0.2		Penicillin binding protein (class B)
yqeB	0.7	0.3	0.2		Putative membrane protein
ytzE	0.6	0.3	0.3		Similar to transcriptional regulator (DeoR family)
***ptb-bcd-buk**-lpdV-bkdAA-bkdAB-bkdB*	1.4	0.3	0.3	CodY/ bkdR/ σ^L	Degradation of the branched-chain amino acids leucine, isoleucine and valine
yppF	0.9	0.4	0.3	Spo0A	Unknown
ecsA	0.9	0.4	0.3		ABC transporter (ATPase)
*yjc**OP**Q*	1.2	0.4	0.3	σ^D	Unknown
yjcM	1.9	0.3	0.2	Spo0A	Similar to alcohol dehydrogenase and to coat-associated protein YhbB
*yybN**ML**K*	1.2	0.5	0.2	Rok	Unknown
yweA	1.8	0.3	0.1		Unknown
ykuV	1.2	0.4	0.3		Thiol:disulfide oxidoreductase, membrane protein

Table 3.2 (Continued)

Gene /Operon[a]	Fold changes			Regulator[b]	Functions[c]
	ΔMWX/ WT	Δ7ECF/ WT	Δ7ECF /ΔMWX		
gntP	1.2	0.5	0.3		Gluconate permease
yclJ	1.2	0.6	0.2		Putative response regulator of YclJK two-component regulatory system.
*yvr**PON***	2.1	0.5	0.2		Similar to ABC transporter

[a.] Genes in operons are shown in bold. The fold change for an operon is the average of fold changes of genes in bold in the table.

[b.] M refers to σ^M, W refers to σ^W, X refers to σ^X, and Y refers to σ^Y.

[c.] Function annotation are mainly based on GenoList (http://genodb.pasteur.fr/cgi-bin/WebObjects/GenoList) (6).

and σ^{YlaC} regulon composition is quite sparse, though we do detect the *sigY* operon and the *ybgB* gene, the only confirmed target operons of σ^{Y} to date (16). Several genes previously identified as putative σ^{Y} or σ^{YlaC} regulon members based on microarray experiments using strains harboring a single IPTG inducible ECF σ gene (either P_{spac}-*sigY* or P_{spac}-*ylaC*) (3) were not detected in our analysis, possibly due to the differences in experimental conditions and strain backgrounds.

Operons affected by σ^{Y} or σ^{YlaC} include: *ysbAB*, *ptb-bcd–buk*, *yjcOPQ*, *yybNMLK*, and *yvrPON*. Several of these genes are known to be regulated by other regulators, such as Spo0A, Rok, and σ^{L} (Table 3.2). Whether their regulation by ECF σ factors (as detected in our experiments) is independent of other regulators is not known. Four genes with annotated functions (*ykuA*, *ecsA*, *ykuV* and *gntP*) and 8 genes of unknown function (*yolA, yolB, yqeB, ytzE, yppF, ybdN, yweA,* and *yclJ*) were also identified as potential σ^{Y} and/or σ^{YlaC} regulon members.

The most dramatic changes in the Δ7ECF strain were a loss of signal for three genes (*immR* (formerly *ydcN*), *yddM* and *yddK*) within the integrative and conjugative element ICE*Bs1* that is inserted into the *trnS-leu2* region (5). This suggested a possible loss of the ICE*Bs1* element in the Δ7ECF strain (Figure 3.1) which was confirmed by PCR: two ICE*Bs1* genes *immR* and *yddE* were easily detected in the WT strain and ΔMWX, but no products were detected in the Δ7ECF strain (data not shown). Since strains Δ7ECF and ΔMWX were constructed in different labs using different methods, we asked whether the loss of ICE*Bs1* was a random incident, or related to the multiple ECF σ factor deletion events in strain Δ7ECF. To address this question, we compared

the excision rates of ICE*Bs1* amongst strains 168 (WT), ΔMWX, ΔVYZ-YlaC, JMA208 (as a positive control) and JMA222 (as a negative control) using linear-range PCR according to (4, 5). No ICE*Bs1* excision events were detected in strains of 168, ΔMWX or ΔVYZ-YlaC, suggesting that excision is not triggered by either of these multiple deletions (data not shown). This result does not exclude the possibility that loss of all seven ECF σ factors might somehow trigger excision. In general, the genes encoded in ICE*Bs1* are mainly involved in ICE*Bs1* excision and transfer (or are of unknown function). Since most of the phenotypes associated with the Δ7ECF strain are shared with the ΔMWX strain (which retains ICE*Bs1*), we infer that these phenotypes are due to the loss of ECF σ factors and not to the loss of the ICE*Bs1* element.

Putative σ^{ECF} target genes. There are 19 genes down-regulated in all three microarray comparisons, indicative of the involvement of ECF σ factors from both groups (MWX and the VYZ-YlaC) in their expression. These genes are assigned as putative σ^{ECF} regulon members. Of these 19 genes, five genes (*yrhI*, *rodA*, *ywtF*, *ythP*, and *ybfO*) have been previously assigned as σ^{MWX} regulon members. Deletion of all 7ECF σ factors reduced their expression compared to the ΔMWX deletion. Another gene (*pel*) is only reduced ~2 fold in expression in strain ΔMWX *versus* WT, with a significant reduction also noted in the comparisons Δ7ECF *versus* WT and Δ7ECF *versus* ΔMWX. The *pel* gene encodes pectate lyase (EC 4.2.2.2), whose transcription is regulated by σ^{A} and repressed by TnrA (40, 50). We could not identify a σ^{MWX}

promoter element upstream of the translational start site of *pel,* suggesting that its regulation by ECF σ factors may be indirect.

Of the 22 putative σ^{ECF} regulon genes, eleven are organized into two operons, the sublancin operon and the *eps* operon. Three genes of the sublancin operon, *sunT*, *bdbA*, and *bdbB* were down-regulated in all three microarray analyses, suggesting complex regulation of this operon. The sublancin operon is located in prophage SPβ, and is involved in the production, modification and secretion of the lantibiotic sublancin 168 (41). Transcription of the sublancin operon is primarily regulated by the transcriptional regulator Abh, whose transcription is largely dependent on σ^{X}, and to a lesser degree on σ^{M} (34). Sublancin production is known to be reduced in strains lacking σ^{X} and σ^{M} and the present analysis reveals an even greater reduction in strain Δ7ECF. This result suggests that sublancin production is not only subject to primary regulation by σ^{X} and σ^{M}, but also by other (VYZ-YlaC group) ECF σ factors. The expression of *abh* was similar in strains Δ7ECF and ΔMWX, suggesting that this additional regulation may be independent of Abh. Consistent with this idea, we previously detected residual expression of sublancin in an *abh* null mutant (34).

The largest operon detected in our microarray analysis was the *eps* operon consisting of genes *epsABCDEFGHIJKLMNO* (formally *yveKLMNOPQRST yvfABCDEF*). Eight genes within this operon exhibited on average 2.5 fold lower expressions in strain ΔMWX *versus* WT, 5 fold lower expression in strain Δ7ECF *versus* WT, and 5 fold lower expression in strain Δ7ECF *versus* strain ΔMWX, suggesting the involvement σ^{ECF} factors in expression. The *eps* operon encodes

exopolysaccharide synthesis enzymes, and is essential for biofilm formation in *B.subtilis* (7). Interestingly, the expression of another major biofilm gene *tasA* was also significantly reduced in all three comparisons. TasA is major protein component of biofilm matrix (9), and has recently found to form amyloid fibers in *B. subtilis* (43). The down-regulation of these biofilm genes suggests that ECF σ factors regulate biofilm formation. Previous work has shown that deletion of *sigX*, as well as a triple deletion of *sigMWX* in an undomesticated strain background (NCIB3610), resulted in reduced biofilm formation (35, 39). The reduced transcription of essential biofilm genes that we note provides a plausible genetic basis for these earlier observations.

Overlapping regulation of ECF σ factors in biofilm formation. Our analysis suggests that multiple ECF σ factors may play a role in biofilm formation. To explore the involvement of ECF σ factors in biofilm formation, WT, ΔMWX, and Δ7ECF strains, and a strain harboring a quadruple deletion of *sigY*, *sigV*, *sigZ*, and *ylaC* (ΔVYZ-YlaC) were tested for their ability to form biofilms using a microtiter plate assay (Figure 3.3). A strain harboring a null mutation in *epsH* (Δ*espH*) was used as negative control. The ΔVYZ-YlaC strain only slightly reduced biofilm formation (P=0.03), while the triple mutant strain ΔMWX was modestly, but reproducibly, reduced in biofilm formation. The most dramatic reduction in biofilm formation was observed with stain Δ7ECF (Fig. 3.3A). These results suggest that σ^{MWX} are major factors regulating biofilm formation, and that $\sigma^{VYZ\ YlaC}$ may have residual and additive effects. Recently Murray *et al.* reported that σ^{X} is involved in controlling biofilm architecture in strain NCIB3610 through Abh, and that over-expression of Abh could compensate for the loss of *sigX* (39). We tested whether over-expression of Abh could

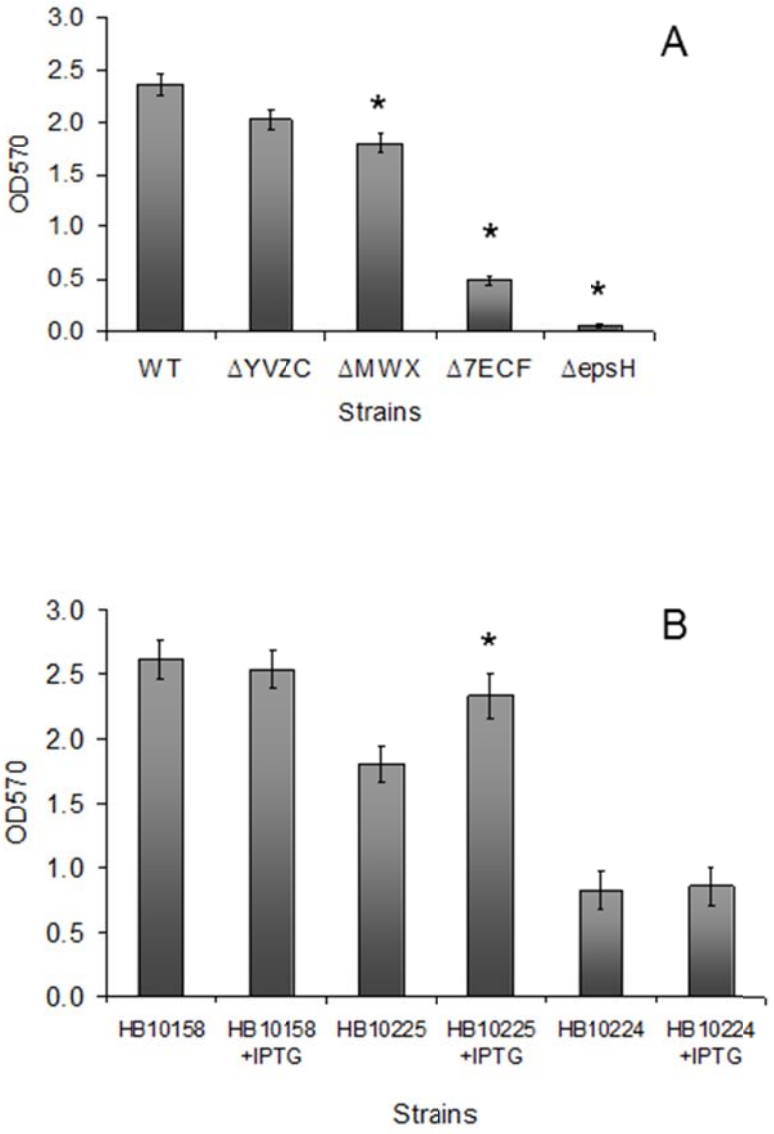


Figure 3.3. Microtiter plate assays of biofilm formation by ECF σ mutants. All strains were assayed after 65h of growth under biofilm formation conditions. The bar graphs show the means and standard errors of the means (error bars). The Student's *t* test was performed, and the statistically significant value with P<0.001 is noted as * above the bar graph. In A), unpaired *t* tests were performed comparing mutants to WT; in B) paired *t* test were used to compare the conditions with and without IPTG induction between the same strains. The strains in use are HB10158 (168 P_{spac}-*abh*), HB10225 (ΔMWX P_{spac}-*abh*) and HB10224 (Δ7ECF P_{spac}-*abh*).

also restore biofilm formation in both strains ΔMWX and Δ7ECF. Over-expression of Abh was able to restore biofilm formation in strain ΔMWX, but not in strain Δ7ECF (Figure 3.3B). There was no growth defect for these strains in the biofilm growth media (data not shown), indicating that these effects are specific for biofilm formation. Together, these results suggest that there are ECF σ target genes, other than Abh, that affect biofilm formation. In light of the large number of cell envelope processes affected by ECF σ factors, this result is perhaps not surprising.

Biolog Phenotype Microarray. ECF σ factors play important roles in maintaining cell envelope integrity and contribute to resistance against cell-envelope acting antibiotics and other chemicals. Single deletions of *sigM, sigW or sigX* alone have shown high susceptibility to a variety of cell envelope stresses (12, 13, 15, 36, 45), and the ΔMWX triple mutant displayed additional sensitivities not seen with the single mutants (35). We hypothesized that the deletion of all 7 ECF σ factors might give rise to additional phenotypes. To test this idea, we employed a phenotype microarray (PM) analysis to screen for phenotypes associated with strains Δ7ECF and ΔMWX. PM analysis is a high throughput approach to screen for novel phenotypic traits linked to genetic alterations (8, 38, 47). Our PM tests included 960 assays for carbon, nitrogen, phosphorus and sulfur source utilization, nutrient stimulation, pH and osmotic stresses, and 240 assays for chemical sensitivities. Similar to the strategy used in the microarray analysis, two PM analyses were performed to screen for the phenotypic differences between strains Δ7ECF and WT, and between strains ΔMWX and WT. Our goal was to detect phenotypes associated with not only σ^{MWX}, but also the lesser understood $\sigma^{VYZYlaC}$ factors. The significant phenotypic differences between strains

ΔMWX *versus* WT and Δ7ECF *versus* WT are summarized in Table S3.1 (supplementary information).

As expected, strain ΔMWX displayed resistance to tetracycline, kanamycin and erythromycin (and related antibiotics doxycycline, chlortetracycline, demeclocycline, rolitetracycline, oleandomycin, troleandomycin, neomycin, and paromomycin) owing to the use of these resistance cassettes to delete the genes encoding ECF σ factors in strain ΔMWX. As strain Δ7ECF carries unmarked deletions (2), this strain did not display these resistances. Relative to the WT strain and to ΔMWX, strain Δ7ECF displayed similar susceptibility to sisomicin (aminoglycoside), and higher susceptibility to chlorpromazine (phenothiazine), and chelerythrine (protein kinase C inhibitor).

In the tests for nutrient utilization, no growth differences were detected in either mutant strain with regards to carbon, phosphorous, and sulphur source utilization. However, utilization of a wide range of amino acids as a nitrogen source was impaired with both mutant strains relative to WT. Original growth kinetics from the PM analysis indicated that both mutant strains either failed to grow or grew poorly under these conditions relative to the WT strain. Although σ^Y has been reported to be induced under nitrogen starvation conditions (46), we did not detect a significant difference between strains ΔMWX and Δ7ECF with regard to nitrogen source utilization in the PM analysis.

We identified a variety of new antibiotics and chemicals that strongly inhibit the growth of strains ΔMWX and Δ7ECF. These antibiotics could be further classified into 3 major categories based on their mode of action: cell envelope inhibitors, DNA

synthesis inhibitors, and toxic ions. To confirm these results, we tested many of these chemicals using disk diffusion assays (Figure 3.4). In addition, we also tested several other antibiotics and chemicals (polymyxin B, bacitracin, fosfomycin, ampicillin, and triton X-100) previously shown to have ECF σ factor-related resistance determinants.

σ^{MWX} are the major ECF σ factors involved in cell envelope stress susceptibility. In the disk diffusion assay, all test compounds, except for protamine sulfate, inhibited the growth of both strains ΔMWX and Δ7ECF relative to the WT strain (Figure 3.4). Protamine sulfate did not inhibit any of the strains (data not shown). It is possible that a different growth condition may be required to detect the susceptibility differences to protamine sulfate. Results from the disk diffusion assays differed from the PM analysis with three chemicals: the antibiotics aztreonam and ofloxacin and $ZnCl_2$. The PM analysis suggested an increase in resistance to these chemicals in strain ΔMWX, but the disk diffusion analysis demonstrated an increase in sensitivity to these chemicals for both strains ΔMWX and Δ7ECF when compared with the WT strain. This may reflect differences in the growth conditions between the PM assay and the disk diffusion assay.

Of the 13 compounds tested by disk diffusion, the most dramatic changes in susceptibility were observed with cell wall acting-antibiotics fosfomycin, bacitracin, aztreonam and cefuroxime. ECF σ factor-dependent resistance to fosfomycin and bacitracin has been previously documented, and two genes, *fosB* (σ^W regulon) (11) and *bcrC* (σ^{MX} regulon) (13) mediate this resistance.

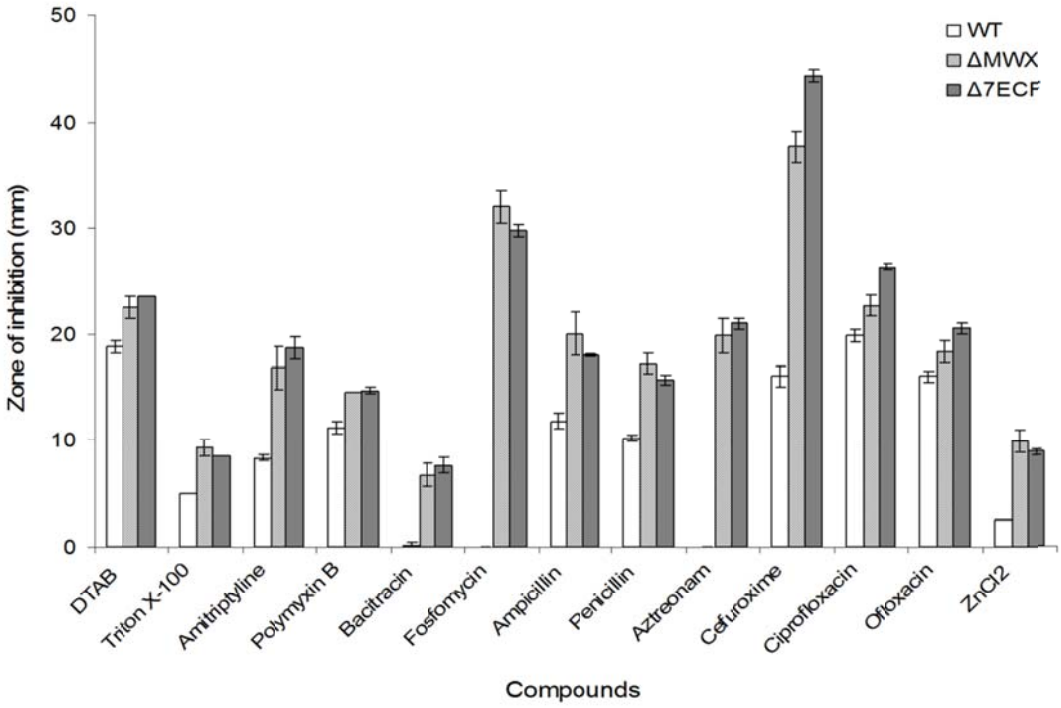


Figure 3.4. Disk diffusion assays of antibiotic sensitivity. Each bar represents the average zone of inhibition of at least three assays performed with biological triplicates of each strain tested. Error bars indicate the standard deviations. The zone of inhibition is expressed as the total diameter of the clear zone minus diameter of the filter paper disk (6.5mm). DTAB, dodecyltrimethyl ammonium bromide.

Aztreonam and cefuroxime are ß-lactam antibiotics that competitively inhibit the peptidoglycan transpeptidase activities of penicillin binding proteins (PBPs) to inhibit cell wall synthesis. Aztreonam specifically inhibits the cell division protein PBP3 in *E.coli* (23), but its target(s) in *B.subtilis* is unknown. Although active against most gram negative bacteria, aztreonam is generally considered ineffective against gram positive bacteria. Consistent with this notion, we did not observe a zone of inhibition using aztreonam against WT cells. However, the deletion of *sigMWX* (as well as the deletion of all 7 ECF σ factors) rendered *B.subtilis* highly sensitive to this drug, suggesting the involvement σ^{MWX} in the bacterium's innate resistance mechanisms. Deletion of *sigMWX* also significantly increased cell susceptibility to cefuroxime even though the WT cells are quite sensitive already. The resistance mechanisms to these two ß-lactam antibiotics and their association with ECF σ factors are unknown, and currently under investigation.

In addition, we found strains ΔMWX and Δ7ECF had increased susceptibility to three other chemicals, including dodecyltrimethyl ammonium bromide (DTAB), amitripyline, and $ZnCl_2$. DTAB is one of the most widely used cationic surfactants in chemical industries (31). Amitripyline is commonly used as antidepressant, and its detergent properties allow partitioning into lipid bilayers, and contribute to its toxicity to mammalian neurons (32). Previous work has already demonstrated that a ΔMWX strain (in a strain NCIB3610 background) is more highly sensitive to the detergents Triton X-10 and SDS (35). In addition, strains ΔMWX and Δ7ECF are more sensitive to the toxic ion Zn. The reason for this effect is unknown. Finally, our data also

confirms previous observations (35) that σ^{MWX} influences resistance to three other cell envelope-acting antibiotics, ampicillin, penicillin G and polymycin B.

For most of antibiotics tested in our analysis, there were no significant sensitivity differences between the ΔMWX and Δ7ECF strains (Figure 3.4) indicating that σ^{MWX} are the major ECF σ factors involved in the cell envelope stress resistance, except for one cephalosporin (cefuroxime) and two DNA topoisomerase inhibitors (ciprofloxacin and ofloxacin). Slightly higher susceptibility to cefuroxime were found in strain Δ7ECF than strain ΔMWX. In the case of ciprofloxacin and ofloxacin, only strain Δ7ECF is significantly susceptible to both drugs. Further disk diffusion tests with the single deletions of each of the *sigVYZ ylaC* factors, or a quadruple mutant did not display difference in susceptibility to these three drugs when compared to WT (data not shown). We suspect that a complex overlapping regulation by some combination of σ^{MWX} and $\sigma^{VYZ\ YlaC}$ factors are required for optimal resistance in WT cells.

3.4 Concluding Remarks

We here describe transcriptomic and phenotypic differences among *B. subtilis* strains lacking key subsets of ECF σ factors. Using microarray comparison experiments, we identified 87 genes controlled by one or more ECF σ under these non-stressed conditions. Many of these are previously known σ^{ECF} target genes, whereas most others of the newly identified target genes lack apparent σ^{ECF} promoters, suggesting that their regulation may be indirect. Indeed, ECF σ factors are known to activate the

expression of several known or putative transcription factors. In addition to confirming and extending previous findings regarding the contribution of ECF σ factors to cell envelope stress resistance, the comparisons here provide evidence for a role of ECF σ factors in biofilm formation and, using phenotypic microarrays, we identify a major role for ECF σ factors in resistance to ß-lactam antibiotics such as aztreonam and cefuroxime.

3.5 Acknowledgements

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The supplementary information (Table S3.1) can be found at the end of this chapter.

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3.6 Supplementary Information

Table S3.1. Phenotype MicroArrray analysis of strains ΔMWX or Δ7ECF *vs.* WT.

Compound [a]	ΔMWX *vs.* WT [b]	Δ7ECF *vs.* WT [b]	Mode of action [c]
Cell envelope stress			
Cefuroxime *	-148		Wall, cephalosporin
Aztreonam *	139		Wall, lactam
DTAB *		-127	membrane, detergent, cationic
Lauryl sulfobetaine	-110	-112	membrane, detergent, zwitterionic
Niaproof	-115	-103	membrane, detergent, anionic
Poly-L-lysine		-80	membrane, detergent, cationic
Amitriptyline *		-127	membrane, transport
Protamine Sulfate *		-113	membrane, ATPase
DNA synthesis			
Lomefloxacin		-130	DNA topoisomerase (GP); fluoroquinolone
Pipemidic Acid		-109	DNA topoisomerase (GP), quinolone
Ciprofloxacin *		-89	DNA topoisomerase, quinolone
Ofloxacin *	120	-88	DNA topoisomerase (GP); fluoroquinolone
Toxic ions			
Thallium (I) acetate	-153	-169	toxic cation
Zinc chloride *	265	120	toxic cation
Sodium Tungstate	-205		transport, toxic anion, molybdate analog
Protein synthesis			
Tetracycline	220		protein synthesis, tetracycline
Doxycycline	267		protein synthesis, tetracycline
Chlortetracycline	336		protein synthesis; 30S ribosomal subunit; tetracycline
Demeclocyline	382		protein synthesis; 30S ribosomal subunit; tetracycline
Rolitetracycline	166		protein synthesis, 30S ribosomal subunit, tetracycline
Erythromycin	663		protein synthesis; 50S ribosomal subunit; macrolide
Oleandomycin	693		protein synthesis, 50S ribosomal subunit, macrolide
Troleandomycin	118		protein synthesis, macrolide
Kanamycin	127		protein synthesis; 30S ribosomal subunit; aminoglycoside
Neomycin	323		protein synthesis; 30S ribosomal subunit; aminoglycoside
Paromomycin	130		protein synthesis, aminoglycoside
Sisomicin	-94	-85	protein synthesis, aminoglycoside

Table S3.1 (continued)

Chlorpromazine		-127	phenothiazine
Chelerythrine		-81	protein kinase C
Nutrient source			
Ala-Ala-Ala	-36	-32	N-source
D-Ala-Leu		-35	N-source
Ala-Gln	-44		N-source
Ala-His	-31		N-source
D;L-a-Amino-N-Butyric Acid	-39		N-source
Gly-Gly-Leu	-33	-31	N-source
Gly-Met	-32		N-source
Leu-Asp		-33	N-source
L-Ile	-58		N-source
L-Ornithine	-33		N-source
L-Trp	-30		N-source
Lys-Ala	-43	-39	N-source
Lys-Leu	-31	-34	N-source
Lys-Val	-39		N-source
Met-Pro		-37	N-source
Phe-Ile	-38		N-source
Phe-Trp	-31	-31	N-source
Pro-Ala		-35	N-source
Pro-Leu	-32	-53	N-source
Pro-Pro	-42	-31	N-source
Pro-Tyr	-52	-39	N-source
Ser-Leu	-43	-53	N-source
Ser-Met	-34	-32	N-source
Ser-Phe	-45	-41	N-source
Ser-Ser	-42		N-source
Ser-Tyr	-46	-30	N-source
Ser-Val	-40		N-source
Thr-Ala	-49	-50	N-source
Thr-Arg	-54	-42	N-source
Thr-Gln	-33	-37	N-source
Thr-Gly	-32	-31	N-source
Thr-Met	-37	-37	N-source
Thr-Pro	-37		N-source
Trp-Ala	-50	-50	N-source
Trp-Arg	-47	-47	N-source

Table S3.1 (continued)

Trp-Asp	-52	-53	N-source
Trp-Glu	-49	-50	N-source
Trp-Gly	-41	-44	N-source
Trp-Lys	-32	-32	N-source
Trp-Ser	-37	-42	N-source
Trp-Trp	-58	-50	N-source
Trp-Tyr	-39	-39	N-source
Tyr-Ala	-45	-33	N-source
Tyr-Gln	-45	-42	N-source
Tyr-Glu	-36	-35	N-source
Tyr-Gly	-38	-30	N-source
Tyr-His	-37	-33	N-source
Tyr-Leu	-52	-53	N-source
Tyr-Phe	-48	-39	N-source
Tyr-Tyr	-34	-34	N-source
Val-Asn	-34	-45	N-source
Val-Asp	-48	-31	N-source
Val-Gly	-53	-52	N-source
Val-Ile	-57	-49	N-source
Val-Leu	-45	-48	N-source
Val-Tyr	-37	-44	N-source
Val-Tyr-Val	-38		N-source
Val-Val	-46	-44	N-source

a. Compounds that are subjected to further susceptibility with disk diffusion assays are noted by *.

b. Growth measurements were done using cell respiration indicator as described in Methods and Materials. Negative values indicate significantly poorer growth of the mutant strain relative to 168 while positive values indicate better growth of the mutant strain relative to 168.

c. Possible effect or mode of action (original Biolog annotation). Of note, not all modes of action are applicable to *B. subtilis.*

CHAPTER 4

ANALYSIS OF THE ROLE OF BACILLUS SUBTILIS SIGMA M IN β-LACTAM RESISTANCE REVEALS AN ESSENTIAL ROLE FOR C-DI-AMP IN PEPTIDOGLYCAN HOMEOSTASIS

The *Bacillus subtilis* extracytoplasmic function (ECF) σ factor σ^M is inducible by, and confers resistance to, several cell envelope acting antibiotics. Here, we demonstrate that σ^M is responsible for intrinsic β-lactam resistance, with σ^X playing a secondary role. Activation of σ^M upregulates several cell wall biosynthetic enzymes including one, PBP1, shown here to be a target for the beta-lactam cefuroxime. However, σ^M still plays a major role in cefuroxime resistance even in cells lacking PBP1. To better define the role of σ^M in β-lactam resistance we characterized suppressor mutations that restore cefuroxime resistance to a *sigM* null mutant. The most frequent suppressors inactivated *gdpP* (*yybT*) which encodes a cyclic-di-AMP phosphodiesterase (PDE). Intriguingly, σ^M is a known activator of *disA* encoding one of three paralogous c-di-AMP cyclases (DAC). Overproduction of the GdpP PDE greatly sensitized cells to β-lactam antibiotics. Conversely, genetic studies indicate that at least one DAC is required for growth with depletion leading to cell lysis. These findings support a model in which c-di-AMP is an essential signal molecule required for cell wall homeostasis. Other suppressors highlight the roles of ECF σ factors in counteracting the deleterious effects of autolysins and reactive oxygen species in β-lactam treated cells.

The results of this study were published in Luo Y and Helmann JD. Molecular Microbiololgy. 2012 Feb; 83(3):623-39.

4.1 Introduction

The bacterial cell envelope is crucial for maintaining cell shape and counteracting turgor pressure and is an important target for many antimicrobial compounds (85). The cell envelope of *Bacillus subtilis* contains a cytoplasmic membrane surrounded by layers of cross-linked peptidoglycan (PG), membrane-associated lipoteichoic acids (LTA), and wall-associated teichoic acids (WTA) (23, 78). PG is a polymer of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) glycan chains cross-linked by peptide sidechains. The newly synthesized lipid-linked NAG-NAM units are polymerized to glycan strands by the action of transglycosylase (TG). Concurrent with, or soon after this polymerization, the peptide side chains on the NAM residue are cross-linked by transpeptidase (TP). Both TG and TP are activities of high molecular weight penicillin binding proteins (HMW PBPs), and they are the targets of moenomycin and the β-lactam antibiotics, respectively (23, 53, 86).

β-lactam antibiotics are characterized by the presence of a β-lactam ring which mimics the D-Ala-D-Ala dipeptide substrate of HMW PBP and inhibits the transpeptidation reaction by covalent modification of the TG active site (53). This inhibition disrupts cell wall biosynthesis, triggers the formation of reactive oxygen species (ROS), and results in cell lysis and death (32, 45, 46). Synthesis and incorporation of new PG glycan strands into the existing cell wall requires close

coordination between the biosynthetic machinery (including HMW-PBPs) and autolytic enzymes that allow the separation of already crosslinked glycan strands. When properly coordinated, the cell grows normally and maintains proper cell shape. Conversely, agents that prevent this coordination by inhibiting TG or TP activities of PBPs, or by activating autolysins, lead to lysis and cell death (Figure 4.1). Several models have been advanced to explain how this coordination occurs, but the existence and precise architecture of the proposed biosynthetic holoenzyme is still unclear (9, 84).

There are three major mechanisms that confer high level β-lactam resistance as described for the Gram positive genera *Staphylococcus* and *Streptococcus* and the Gram negative species *Escherichia coli* and *Pseudomonas* spp. These are: (i) expression of β-lactamase(s) that inactivate the antibiotics; (ii) expression of mutated or mosaic PBP alleles that have low affinity for β-lactams; and (iii) the expression of a β-lactam specific efflux pump (67, 87). *B. subtilis* displays a significant level of intrinsic resistance against a variety of β-lactam antibiotics, but the underlying mechanisms are poorly understood. Although there are three putative β-lactamase genes (*penP*, *ybbE,* and *yblX*) in the genome, no β-lactamase activity is detected in the growing cells or supernatant (16). No penicillin-insensitive PBP alleles have been identified nor does an efflux pump-based mechanism appear to be applicable to *B. subtilis* and other Gram positive bacteria. Therefore, the molecular basis of this intrinsic, moderate level β-lactamase resistance is unclear. Recent results suggest that extracytoplasmic function (ECF) σ factors play a role in resistance to β-lactam antibiotics: a triple mutant (strain *sigMWX*) as well as a mutant lacking all 7 ECF σ

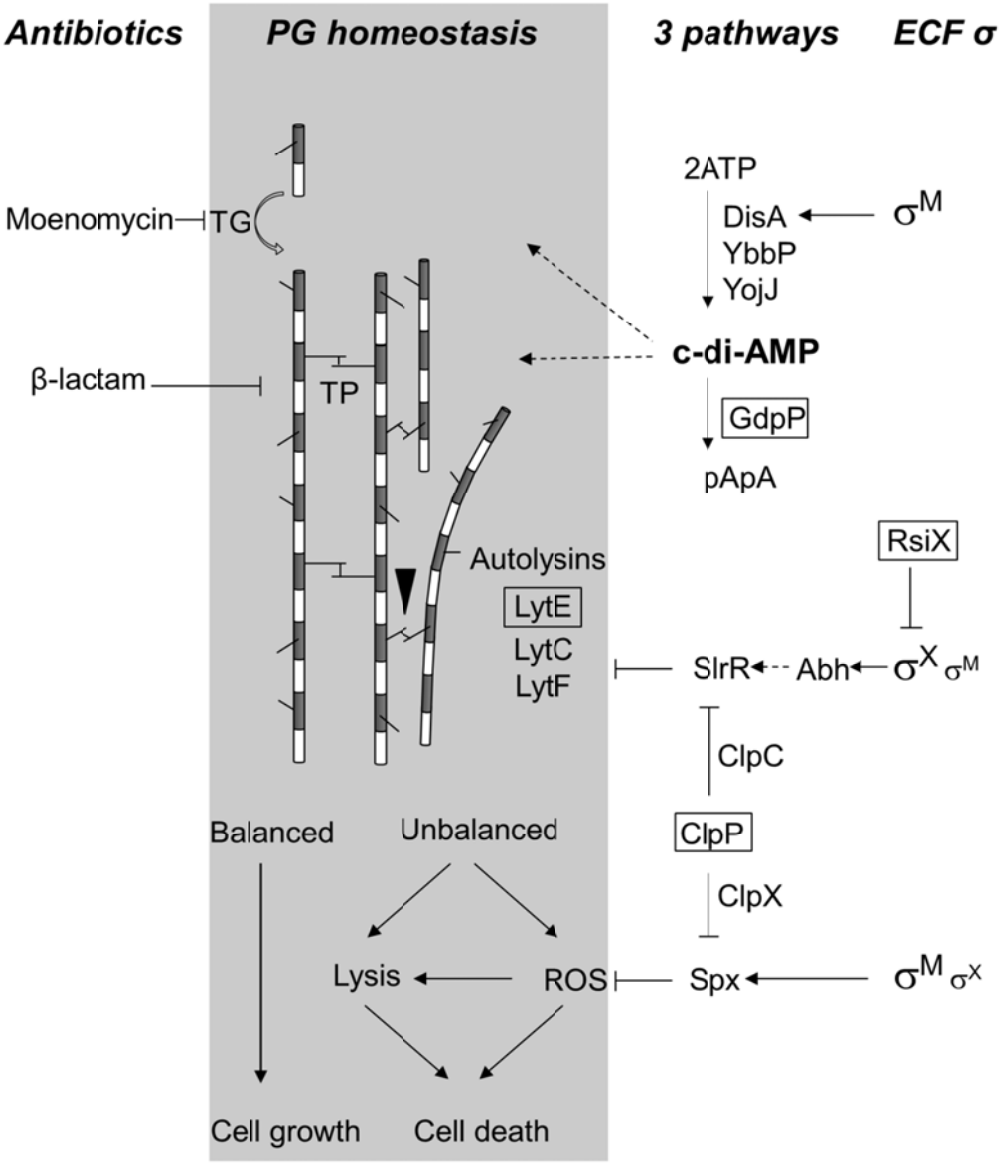

Figure 4.1. Model of peptidoglycan (PG) homeostasis and the contributions of σ^M and σ^X to cell wall antibiotic resistance. The alternating grey and white bars represent N-acetylmuramic acid and N-acetylglucosamine, respectively, which comprise the glycan chains. Peptide crosslinks between strands are introduced by transpeptidation (TP) and are broken by autolytic endopeptidases (black triangle). Moenomycin targets the transglycosylation (TG) step in glycan chain elongation while ß-lactams inhibit TP-mediated crosslinking. The results reported herein, combined with previous results (see text), indicate that σ^M and σ^X contribute to antibiotic resistance by three distinct pathways as shown on the right. Genes identified by Tn7 mutagenesis are boxed. ROS, reactive oxygen species; straight arrow, direct positive regulation; dashed arrow, indirect positive regulation; —| negative regulation.

factors (strain Δ7ECF) is sensitive to β-lactam antibiotics including ampicillin, penicillin G, aztreonam, and cefuroxime (51, 55).

B. subtilis harbors 7 ECF σ factors, σ^M, σ^X, σ^W, σ^V, σ^Y, σ^Z and σ^{YlaC}. Of these, the physiological roles of σ^M, σ^W, σ^X, and more recently σ^V, have been well characterized, and their target regulons have been defined (21, 30, 35, 41). Both expression and activity of these ECF σ factors are often stimulated by cell wall-active antibiotics. σ^M is strongly induced by vancomycin and moenomycin, and confers resistance to moenomycin (21, 55, 83). Activation of the σ^W regulon contributes to resistance to fosfomycin, sublancin, and a toxic peptide SdpC (5, 8). The σ^X regulon is involved in the resistance to nisin and other cationic antimicrobial peptides (6, 7). Finally, σ^V is induced by and provides resistance to lysozyme (30, 36).

In this study, we investigated the roles of ECF σ factors in providing intrinsic resistance to β-lactam antibiotics and, in particular, to cefuroxime (CEF). We found that σ^M plays a primary role in β-lactam resistance, with σ^X as a secondary determinant. We identified Tn7 insertions mutations that restored CEF resistance to a *sigM* mutant. Genetic analysis reveals a central role for the recently identified signal molecule cyclic-di-AMP (c-di-AMP), synthesized in part by a σ^M-activated diadenylate cyclase (DAC), in cell wall homeostasis. In addition, our results highlight the key role of previously defined pathways by which ECF σ factors regulate autolysin activity and resistance to reactive oxygen species.

4.2 Material and Methods

Bacterial strains and growth conditions. B. subtilis strains used are derivatives of strain168 (*trpC2*) and are shown in Table 4.1. Escherichia coli strain DH5α was used for standard cloning procedures. Bacteria were grown in Luria-Bertani (LB) (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) broth at 37^{o}C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg/ml ampicillin for E. coli, and 1 μg/ml erythromycin plus 25 μg/ml of lincomycin (MLS, macrolide-lincomycin-streptogramin B resistance), 10 μg/ml chloramphenicol, 100 μg/ml spectinomycin (Spc), 5 μg/ml tetracycline and 10 μg/ml kanamycin for B. subtilis. OD_{600} readings were taken on a Spectronic 21 spectrophotometer.

Strain Constructions. Gene deletions were generated by replacing the coding region with an antibiotic resistance cassette using long flanking homology PCR (LFH-PCR) followed by DNA transformation as previously described (56). Chromosomal DNA transformations were performed as described previously (34).

The IPTG inducible constructs were generated using vector pPL82 (70). PCR products were amplified from *B. subtilis* 168 chromosomal DNA, digested with endonucleases, and cloned into pPL82. pPL82 contains a chloramphenicol resistance cassette, a multiple cloning site downstream of the $P_{spac(hy)}$ promoter, and the *lacI* gene between the two arms of the *amyE* gene. Primer pairs used for PCR amplification are 5249/5250 for *disA*, 5252/5253 for *ybbP*, 5255/5256 for *yojJ*, 5244/5245 for *gdpP*, and 5244/5258 for $gdpP_{1\text{-}303}$. All oligonucleotide sequences are listed in SI Table S1.

The sequences of the inserts were verified by DNA sequencing (Cornell DNA sequencing facility). pPL82- *gdpP*$_{D420A}$ was generated using overlap joining PCR with pPL82-*gdpP* as DNA template. Primer pairs 5244/5293, and 5294/5245 were first used to amplify the up and down fragments of *gdp*, respectively. The *gdpP*$_{D420A}$ mutation was generated using primers 5293 and 5294. A joining PCR was then performed with the up and down fragments as template and primer pairs 5244/5245. The PCR product was cloned into pPL82 as above, and the insert was verified by DNA sequencing. Plasmids were linearized by ScaI and used to transform *B. subtilis*, where they integrated into the *amyE* locus.

Table 4.1. Strains used in this study

Strain[1]	Genotype	Reference / construction[2]
168	*trpC2*	lab strain
CU1065	*trpC2 attSPβ*	lab strain
PS832	Prototrophic revertant of strain 168	lab strain
BSU2007	168 *sigMWXYZV ylaC* (Δ7ECF)	(1)
HB0031	CU1065 *sigM::kan*	(8)
HB10216	168 *sigM::kan*	chrDNA of HB0031 -->168
HB10016	168 *sigM::tet*	(52)
HB10103	168 *sigX::kan*	(52)
HB10102	168 *sigW::mls*	(52)
HB10114	168 *sigX::kan, sigW::mls*	(52)
HB10117	168 *sigM::tet, sigW::mls*	(52)
HB10113	168 *sigM::tet sigX::kan*	(52)
HB7007	CU1065 *sigX::spc*	(37)
HB15815	168 *sigM::kan sigX::spc*	chrDNA of HB7007 --> HB10216
HB10107	168 *sigM::tet, sigX::kan sigW::mls*	(52)
HB10236	168 *sigZ::kan sigV::cat sigY::mls ylaC::spc*	(51)
HB5421	CU1065 *amyE::P_{sigX}-lacZ cat*	Lab strain
HB5422	CU1065 *amyE::P_{sigW}-lacZ cat*	Lab strain
HB5423	CU1065 *amyE::P_{sigM}-lacZ cat*	Lab strain
HB10183	168 *amyE::P_{sigM}-lacZ cat*	chrDNA of HB5423 --> 168
HB10184	168 *amyE::P_{sigX}-lacZ cat*	chrDNA of HB5421 --> 168
HB10185	168 *amyE::P_{sigW}-lacZ cat*	chrDNA of HB5422 --> 168
PS2062	PS832 *ponA::spc*	(68)
HB10386	168 *ponA::spc*	chrDNA of PS2062 --> 168
HB0047	CU1065 *rsiX::spc*	lab strain
HB10118	168 *rsiX::spc*	chrDNA of HB0047 --> 168
HB10379	168 *sigM::tet rsiX::spc*	chrDNA of HB10118 --> HB10016
HB10536	CU1065 *sigX rsiX::kan*	LFH -->CU1065
HB10378	168 *sigM::tet sigX rsiX::kan*	chrDNA of HB10536 -->HB10016
HB10131	168 *abh::spc*	(52)
HB4728	CU1065 *spx::spc*	lab strain
HB10328	168 *spx::spc*	chrDNA of HB4728 --> 168
HB10348	168 *spx::mls*	LFH -->168
HB10329	168 *sigM::kan spx::spc*	chrDNA of HB4728 --> HB10216
HB15808	168 *sigM::kan abh::spc*	chrDNA of HB10131 --> HB10216
HB15811	168 *sigM::kan abh::spc spx::mls*	chrDNA of HB10348--> HB15808
HB10316	168 *clpP::tet*	LFH-->168
HB10332	168 *spx::spc clpP::tet*	chrDNA of HB10316 --> HB10328

Table 4.1 (continued)

HB10320	168 *sigM::kan clpP::tet*	chrDNA of HB10316 --> HB10216
HB15814	168 *sigM::kan spx::spc clpP::tet*	chrDNA of HB10316 --> HB10329
HB15816	168 *sigM::kan sigX::spc clpP::tet*	chrDNA of HB10316 --> HB15815
HB15823	168 *sigM::kan sigX::spc spx::mls*	chrDNA of HB10348 --> HB15815
HB15824	168 *sigM::kan sigX::spc spx::mls clpP::tet*	chrDNA of HB10316 --> HB15823
HB10278	168 *amyE:: $P_{spac(hy)}$- gdpP cat*	pPL82-*gdpP* -->168
HB10287	168 *amyE::$P_{spac(hy)}$- gdp_{1-303} cat*	pPL82-*$gdpP_{1-303}$* -->168
HB10309	168 *amyE::$P_{spac(hy)}$- $gdpP_{D420A}$ cat*	pPL82- *$gdpP_{D420A}$* -->168
HB10352	168 *gdpP::mls*	LFH -->168
HB10257	168 *sigM::kan gdpP::mls*	chrDNA of HB10352 --> HB10216
HB10295	168 *sigM::kan gdpP::mls amyE::$P_{spac(hy)}$-gdpP cat*	chrDNA HB10278--> HB10257
HB10298	168 *sigM::kan gdpP::mls amyE::$P_{spac(hy)}$-$gdpP_{1-303}$ cat*	chrDNA HB10287 --> HB10257
HB10310	168 *sigM::kan gdpP::mls amyE::$P_{spac(hy)}$-$gdpP_{D420A}$ cat*	chrDNA HB10309 --> HB10257
HB10353	168 *disA::spc*	LFH -->168
HB10334	168 *ybbP::tet*	LFH -->168
HB10335	168 *yojJ::kan*	LFH -->168
HB10365	168 *disA::spc amyE:: $P_{spac(hy)}$- gdpP cat*	chrDNA of HB10278 --> HB10353
HB10366	168 *ybbP::tet amyE:: $P_{spac(hy)}$- gdpP cat*	chrDNA of HB10278 --> HB10334
HB10367	168 *yojJ::kan amyE:: $P_{spac(hy)}$- gdpP cat*	chrDNA of HB10278 --> HB10335
HB10354	168 *disA::spc yojJ::kan*	chrDNA of HB10353 -->HB10335
HB10356	168 *ybbP::tet yojJ::kan*	chrDNA of HB10334 -->HB10335
HB10281	168 *amyE::$P_{spac(hy)}$-disA cat*	pPL82-disA -->168
HB10283	168 *amyE::$P_{spac(hy)}$-ybbP cat*	pPL82-ybbP -->168
HB10285	168 *amyE::$P_{spac(hy)}$-yojJ cat*	pPL82-yojJ -->168
HB10357	168 *disA::spc amyE:: $P_{spac(hy)}$-disA cat*	chrDNA of HB10353 --> HB10281
HB10358	168 *ybbP::tet amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10334 --> HB10283
HB10374	168 *ybbP::tet amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10334 --> HB10285
HB10359	168 *disA::spc ybbP::tet amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10353 --> HB10358
HB10360	168 *disA::spc ybbP::tet amyE:: $P_{spac(hy)}$-disA cat*	chrDNA of HB10334 --> HB10357
HB10375	168 *disA::spc ybbP::tet amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10353 --> HB10374
HB15802	168 *ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$-ybbP cat*	chrDNA of HB10358 --> HB10356
HB15803	168 *ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$-yojJ cat*	chrDNA of HB10374 --> HB10356
HB15801	168 *disA::spc ybbP::tet yojJ::kan amyE:: $P_{spac(hy)}$--disA cat*	chrDNA of HB10354 --> HB10360

Table 4.1 (continued)

HB15806	168 *disA::spc ybbP::tet yojJ::kan amyE::* $P_{spac(hy)}$*-ybbP cat*	chrDNA of HB10353 --> HB15802
HB15807	168 *disA::spc ybbP::tet yojJ::kan amyE::* $P_{spac(hy)}$*-yojJ cat*	chrDNA of HB10353 --> HB15803
HB10209	168 *sigM::tet spo0A::Tn7*	WT Tn7 library --> HB10016
HB10210	168 *sigM::tet tagA::Tn7*	WT Tn7 library --> HB10016
HB10253	168 *sigM::kan gdpP::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10247	168 *sigM::kanrsiX::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10248	168 *sigM::kan lytE::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10246	168 *sigM::kan pbpX::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10273	168 *sigM::kan ymdB::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10249	168 *sigM::kan kinD::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10245	168 *sigM::kan qoxAB:Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10274	168 *sigM::kan ssrA::Tn7*	*sigM::kan* Tn7 library --> HB10216
HB10263	168 *sigM::kan gdpP::mls lytE::Tn7*	*sigM::kan* Tn7 library --> HB10257
HB10264	168 *sigM::kan gdpP::mls clpP::Tn7*	*sigM::kan* Tn7 library --> HB10257

[1] Some genes have multiple Tn7 insertion positions. Only one representative strain number for each gene is listed here.

[2] The donor DNA and recipient strain of transformation are indicated before and after the arrows, respectively.

Antibiotic susceptibility tests. Susceptibility tests for antibiotics were conducted using disk diffusion assay and minimal inhibitory concentration (MIC) test. Mueller Hinton (MH, Sigma-Aldrich) medium was used for both assays. Disk diffusion assays were performed as previously described (51). The bottom agar is 15 ml MH broth supplemented with1.5% agar, and the top agar is 4 ml MH broth supplemented with 0.75% agar. We used BBL™ Sensi-Disc™ Susceptibility Test Discs (BD; cefixime 5 μg, cefoxitin 30 μg, ceftriaxone 30 μg, ceftazidime 30 μg, cefoperazone 75 μg, amoxicillin 30 μg, ampicillin 10 μg, piperacillin 100 μg, oxacillin 1 μg, piperacillin 100 μg, imipenem 10 μg, meropenem 10 μg, and Isoniazid 1μg) and also prepared disks using Whatman filter paper disks (7 mm in diameter) and freshly made stocks of antibiotics (aztreonam 30 μg, cefuroxime 6 μg , penicillin G 10 U, nalidixic acid 30 μg, novobiocin 250 μg, vancomycin 30 μg, polymycin B 250 μg, and moenomycin 50 μg). The zone of growth inhibition was measured after overnight growth at 37°C. For MIC test, fresh single colonies were first grown in MH broth to an OD_{600} of 0.4, and diluted 1:100 in MH broth, and 200 μl of the diluted culture was dispensed in Bioscreen 100-well microtiter plate. Growth was measured spectrophotometrically (OD_{600}) using a Bioscreen incubator (Growth Curves USA, Piscataway, NJ) at 37°C with vigorous shaking. The absorbance was recorded every 30 minutes for 24 hours. Inhibition was defined as a final $OD_{600}<0.2$ at the 12 hour time point (after 12 h, suppressor mutants started to grow up). All antibiotics susceptibility tests were performed with biological triplicates and repeated at least twice.

Bocillin-FL competitive labeling assay. The bocillin-FL labeling assay was performed as previously described (42, 91) with modifications. Overnight cultures of

B. subtilis cells in LB were diluted 1:100 into 5 ml fresh LB broth, and incubated at 37°C with vigorous shaking. When cell cultures reached mid-log phase (OD_{600} 0.4), the cultures were treated with either 0.05 μg/ml (final conc.) of bocillin-FL, or with additional challenge of 0.00625μg/ml (final conc.) of CEF, or an additional 5 μg/ml aztreonam (final conc.) for 10 min. The cells were pelleted by centrifugation and kept at -20°C overnight. The pellet was thawed on ice and resuspended in 50 μl 0.85% NaCl. The cell resuspension was boiled for 5 min with SDS loading buffer, and proteins were separated by 4~12% SDS-PAGE. To visualize the labeled PBPs, the gels were scanned with a Molecular Dynamics Typhoon PhosporImager (excitation at 488 nm and emission at 530 nm), and the images were analyzed using ImageQuant TL (Amersham Biosciences).

Tn7 mutagenesis. The Tn7 mutagenesis libraries were generated with chromosomal DNA using *in vitro* transposition as described (4). The library DNA was transformed into WT *B. subtilis* or a *sigM* mutant strain (HB10216), and the resulting transposants were grown in the presence of 100 μg/ml spectinomycin (Spc) with and without xylose (final concentration of 1%). Chromosomal DNA was prepared from these cultures using phenol-chloroform extraction (76) and considered an amplified Tn7 library. The amplified Tn7 library DNA was transformed into the *sigM* mutant strains (HB10016 or HB10216), and cells were plated on LB agar supplemented with 100 μg/ml Spc, 1% xylose and 2 μg/ml CEF (32 x MIC of the *sigM* strain). Resulting transformants were streaked onto the same selection plate twice. In order to confirm that the increased CEF resistance was due to the presence of the transposon, we performed linkage tests by transforming the chromosomal DNA of the Tn7 mutants into the *sigM* mutant again

and selecting with 100 μg/ml Spc. The resulting transformants (20 colonies for each strain) were then streaked on LB agar supplemented either with 100 μg/ml Spc or with 100 μg/ml Spc plus 2 μg/ml CEF. The transformants that can grow on both plates were counted as linked mutants, and strains with 100% linkage were subjected to Tn7 insertion position mapping using arbitrary PCR as previously described (4). The dependence on xylose was tested by streaking cells on LB agar supplemented with 2 μg/ml CEF or with 2 μg/ml CEF plus 1% xylose. Tn7 mutagenesis with strain *sigM gdpP* (HB10257) was performed as described above, except that 4 μg/ml of CEF (MIC of the WT strain, and 64 x MIC of the *sigM* strain) was used for selection.

β-galactosidase activity test. Strains harboring ECF σ promoter-*lacZ* fusions were grown overnight in LB broth containing appropriate antibiotics and diluted 1:100 into 5 ml LB medium. The culture was grown at 37^{o}C with vigorous shaking to OD_{600}~0.4 (mid-log growth phase), and then split into two aliquots. One was challenged with 8 μg/ml of CEF and the other was untreated. The cultures were returned to 37^{o}C, and samples were collected after 30 min. β-galactosidase assays were performed as described by Miller (26), and each strain was tested in biological triplicates and repeated three times. Data were reported as the mean and SE.

5' rapid amplification of cDNA ends (5' RACE). The transcriptional start site of *ybbP* was determined using 5' rapid amplification of cDNA ends (5'-RACE). Five micrograms of total RNA from a mid-log-phase LB culture was reversed transcribed to cDNA using TaqMan reverse transcription reagents (Roche) and oligo *ybbP*-rev-GSP3 (5584) as primer. The 3' end of cDNA was tailed with poly-dCTP using terminal

deoxynucleotidyl transferase (New England Biolabs). The tailed cDNAs were then amplified by PCR with primers AAP (3314) and *ybbP*-rev-GSP4 (5585). The PCR products were subjected to DNA sequencing (Cornell DNA sequencing facility).

Growth rate test. Fresh single colonies were first grown in MH broth to OD_{600} of 0.4, and diluted 1:100 in MH broth, and inoculated in Bioscreen microtiter plates with a total inoculum of 200 µl. Growth was measured spectrophotometrically (OD_{600}) using a Bioscreen incubator (Growth Curves USA, Piscataway, NJ) at 37°C with vigorous shaking. The specific growth rate of each strain was calculated from the exponential growth phase. Each test was performed with biological triplicates and repeated twice.

Depeletion of c-di-AMP and microscopic imaging. Strain HB10359 was grown in MH broth supplemented with 1mM IPTG to mid-exponential phase, and collected by centrifugation. The cells were washed twice with MH medium, and resuspended to OD_{600} of 0.2 in fresh MH broth, or MH broth supplemented with 1mM IPTG, SMM (20 mM $MgCl_2$, 10% sucrose, 20 mM maleic acid, pH 7.0), 10% sucrose, or 10mM $MgSO_4$. 200 µl of each cell resuspension was added a Bioscreen microtiter plate, and incubated at 37°C with vigorous shaking. For phase contrast and fluorescence microscopy, 1µg/ml (final concentration) of cell membrane stain FM 4-64 (Invitrogen) was added to the cell culture, and incubated at 37 °C for 30min with shaking. 5 µl of cells were then mounted on microscope slide coated with a thin film of 1% agarose as previously described in (27). Microscopy was performed using an Olympus BX61 epifluorescence microscope. Images were acquired using Cooke SensiCam and Slidebook software (Intelligent Imaging Inc.).

4.3 Results and Discussion

σ^M is the major ECF σ factor involved in the intrinsic resistance to cefuroxime. Previously, we showed that a null mutant lacking all 7 ECF σ factors (strain Δ7ECF) has higher sensitivity to numerous antibiotics (including several β-lactams) compared to the wild type (WT) strain (51). To clarify the role of ECF σ factors in mediating the intrinsic resistance to β-lactam antibiotics, we here sought to identify both the ECF σ factor(s) and the relevant pathways responsible for resistance using cefuroxime (CEF) as a model ß-lactam.

Isogenic strains carrying single or multiple mutations in genes encoding ECF σ factors were tested for CEF susceptibility using disk diffusion and minimal inhibition concentration (MIC) assays. A *sigM* null mutant showed elevated sensitivity to CEF, whereas other single mutants showed little or no change (Figure 4.2). The double *sigM sigX* mutant displayed high sensitivity equivalent to the Δ7ECF strain. A *sigW* mutant showed no effect, although effects on ß-lactam resistance have been seen in other *B. subtilis* strain backgrounds (49). None of the other four ECF σ factors played a role in CEF resistance, even when a multiple mutant strain was tested (Figure 4.2). We conclude that σ^M is the major ECF σ involved in the intrinsic resistance to CEF, with σ^X playing a secondary role apparent in strains lacking σ^M. These results suggest that the major resistance pathway(s) depend exclusively on σ^M for their expression, with one or more additional pathways that can be activated by either σ^M or σ^X (as revealed in the double *sigM sigX* mutant). As described previously, several ECF σ factor promoters can be recognized by more than one ECF σ factor (38, 55, 69). As we will

show later in this study, genes encoding the transcription factors Abh and Spx are recognized by both σ^M and σ^X and are involved in CEF resistance.

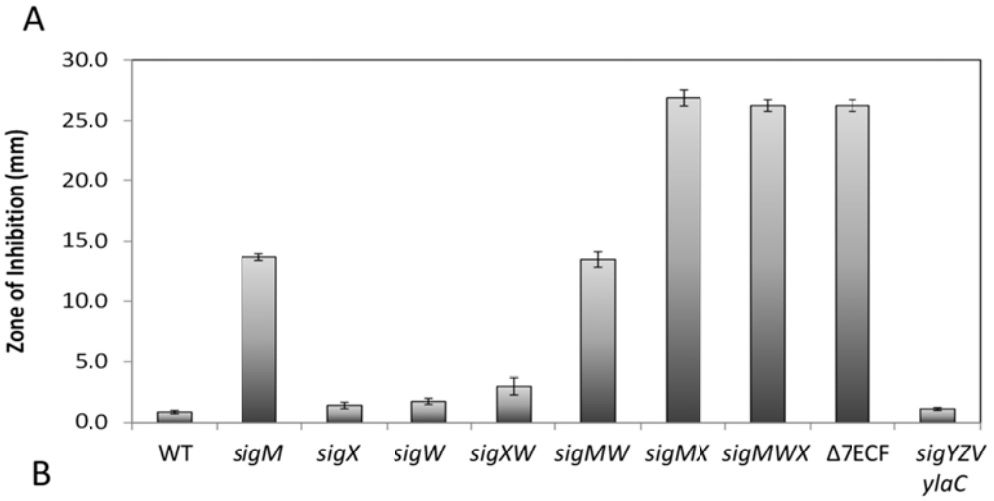

Figure 4.2. σ^M is the major ECF σ involved in the intrinsic resistance to CEF and σ^X plays a secondary role. **(A)** The susceptibility of each strain was tested using disk diffusion assay with 6 μg CEF. The zone of inhibition is expressed as the total diameter of the clearance zone minus the diameter of filter paper disk (7mm). The means and SE from at least 3 biological replicates are reported. **(B)** MIC values are shown under the bar graph.

Antibiotic resistance pathways are often transcriptionally activated in the presence of the cognate antibiotic. ECF σ factors typically autoregulate their own expressions and we and others have previously characterized the relevant autoregulatory promoters (2, 35, 83). We therefore monitored the effect of CEF on expression from the autoregulatory promoters for *sigM*, *sigW*, and *sigX*. In each case, a 2~3 fold induction was observed (Table 4.2). In contrast, low (basal) activity and no induction were detected for the other four ECF σ factors (*sigY, sigV, sigZ, ylaC*) (data not shown). This induction profile is consistent with prior results demonstrating that σ^M, σ^X and σ^W are responsive to cell envelope stress and are activated by an overlapping set of inducers (21, 33, 55, 58).

Table 4.2. ECF σ promoter activities induced after treatment with 8 μg/ml CEF for 30 min. Activities (Miller Units) were measured using β-galactosidase assays and the means and SE are reported.

Reporter fusion	**Untreated**	**CEF treated**
P_{sigM}-lacZ	3.7±0.5	10.1±0.5
P_{sigX}-lacZ	38.6±1.3	99.7±3.5
P_{sigW}-lacZ	35.6±1.9	71.3±2.3

CEF targets PBP1, 2a, 2b and 4. The σ^M regulon is known to include several enzymes involved in various aspects of cell wall synthesis including one HMW PBP (PBP1, encoded by *ponA*) (21). In most cases, σ^M-dependent promoters serve to up-regulate gene expression in response to stress, but are not solely responsible for expression due to the presence of other promoters. In the case of *ponA*, this gene can be transcribed from two promoters: one is σ^M dependent, and the other is σ^A-dependent. We here hypothesized that one mechanism of resistance might be the σ^M-dependent upregulation of PBP1 or other factors involved in assembly or function of cell wall biosynthetic complexes.

To identify the targets of CEF, we performed bocillin-FL competitive labeling assays (42, 91). Five HMW PBPs (PBP1, 2a, 2b, 2c, 4) and one low molecular weight penicillin-binding protein (LMW PBP) (PBP5) were detected by bocillin-FL labeling and CEF competed with bocillin-FL for binding to PBP1, 2b, 2c and 4 (Figure 4.3). Since only six PBPs can be detected in this assay, it is possible that other PBPs are also targets for CEF. No differences in either PBP profile or relative affinity for CEF binding were apparent in a comparison of the CEF sensitive *sigMWX* mutant and the WT strain using the bocillin-FL labeling assay (data not shown). This suggests that mutants lacking ECF σ factors are not altered in their CEF susceptibility due to a gross change in the levels of PBPs.

Since PBP1 is a target for CEF, we hypothesized that σ^M-mediated upregulation of PBP1 might contribute to β-lactam resistance. However, deletion of *ponA* did not alter CEF susceptibility (a *ponA* null mutant and WT have an identical zone of inhibition). Thus, upregulation of PBP1 by σ^M does not appear to be a major

mechanism of CEF resistance. We next tested whether *B. subtilis* expresses β-lactamase using the chromogenic substrate nitrocefin (74). No β-lactamase activity could be detected (either prior to or after CEF treatment) in the WT, *sigMWX* or Δ7ECF strains (data not shown). Thus, the role of ECF σ factors in CEF resistance does not appear to be due to alterations in CEF targets or due to degradation by β-lactamases.

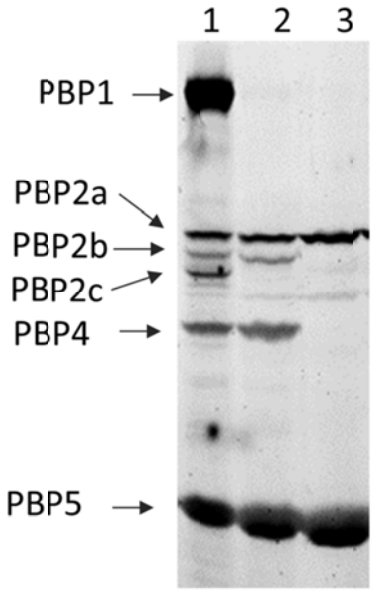

Figure 4.3. CEF binds to PBP 1, 2b, 2c, and 4. PBPs in vegetatively growing cells were labeled with bocillin-FL (lane 1). The binding of bocillin-FL to PBPs was subjected to competitive inhibition by the addition of aztreonam (Lane 2) or CEF (Lane 3). Proteins were separated by 4~12% gradient SDS-PAGE, and visualized by using a Typhoon Fluorimager.

Tn7 mutagenesis reveals multiple pathways involved in CEF resistance. To gain insights into the pathways contributing to CEF resistance, we performed Tn7 transposon mutagenesis and selected for mutations that restored CEF resistance to a *sigM* mutant. The Tn7 transposon derivative we used harbors an outward-facing, xylose-inducible promoter which thereby allows recovery of both loss of function (gene disruption) and gain of function (xylose-dependent up-regulation) mutations (4). Insertion libraries were generated *in vitro* using WT genomic DNA as a target and then transformed into competent *B. subtilis* cells with selection for both the transposon (spc^R) and CEF resistance. In an initial study, we recovered numerous insertions linked to *sigM.* In these strains, a functional copy of *sigM* had been co-transformed into the recipient cells. Although this result confirms the importance of σ^M in CEF resistance, it was otherwise uninformative. Therefore, all subsequent experiments used a Tn7 mutant library generated in a *sigM* mutant (HB10216) background.

A total of 520 CEF resistant colonies were obtained in 10 separate experiments. DNA sequence analysis identified 25 unique insertions localized to 10 different genes (Table 4.3). All of the insertions increased CEF resistance in a *sigM* mutant, although none restored resistance to WT levels (Table 4.3). The most frequently observed insertion occurred in *yybT,* an ortholog of a gene recently renamed *gdpP* (see below). We therefore performed an additional round of selection, transforming the s*igM* Tn7 library into a *sigM gdpP* double mutant strain (HB10257). This selection led to the recovery of insertions in two genes (*lytE* and *clpP*). Both triple mutants (*sigM gdpP lytE*::Tn7 and *sigM gdpP clpP*::Tn7) were at least as CEF resistant as WT (Table 4.3). These results indicate that *gdpP* likely affects a different resistance pathway than *lytE*

and *clpP*. Although our selection plates contained xylose, in no case was CEF resistance dependent on xylose suggesting that in each case we have recovered gene disruption mutations that lead to increased CEF resistance.

Table 4.3. Tn7 insertions that can restore CEF resistance in a *sigM* or a *sigM gdpP* mutant.

Tn7 Mutants	**Unique insertions**	**Gene annotation**	**Resistance to CEF[a]**	**Growth rate relative to WT (%)[b].**
Insertions in a sigM background				
gdpP::Tn7	11	phosphodiesterase	++	98
rsiX::Tn7	1	anti-σ^X	++	100
lytE::Tn7	1	autolysin	++	98
pbpX::Tn7	1	penicillin-binding endopeptidase X	++	95
tagA::Tn7	1	wall teichoic acid biosynthesis	++	81 *
ymdB::Tn7	1	Regulate expression of SlrR	+	68 *
kinD::Tn7	1	negative regulator of Spo0A~P	+	95
spo0A::Tn7	2	initiation of sporulation	+	102
qoxAB:Tn7	3	cytochrome aa3-600 quinol oxidase	+	52 *
ssrA::Tn7	1	transfer-messenger RNA (tmRNA)	+	79 *
Insertions in a sigM gdpP background				
lytE::Tn7	2	autolysin	+++	96
clpP::Tn7	2	ATP-dependent Clp protease proteolytic subunit	++++	81 *

[a] The resistance to CEF was tested using disk diffusion assay with biological triplicates, and repeated twice. The zone of inhibition (mean ± SE) was used for the score. The resistance level of wt is defined as "+++", and Δ*sigM* is "-".

[b] The *sigM* strain has the same growth rate as WT (100%). Strains with noticeably reduced growth rates are labeled with *.

Genes identified in this suppressor mutation are involved in a variety of pathways and functions (Table 4.3). We categorized them into three groups using two criteria: (i) direct or indirect involvement in cell wall metabolism, and (ii) mild or strong effect on CEF resistance. The first group included several insertions that inactivated genes directly involved in cell wall metabolism including *lytE, pbpX, tagA,* and *ymdB*. LytE is a major autolytic endopeptidase in vegetative cells (54, 80). LytE interacts with the actin-like protein MreBH along the cylindrical part of cell wall and with FtsZ and PBP2b at the division septum. It is, therefore, closely related to cell wall synthesis (10). The inactivation of *lytE* presumably increases ß-lactam resistance by delaying cell lysis. PbpX is a LMW PBP that is located at the septum during vegetative growth (77). Its function is unknown, although it was shown previously to be activated by σ^X (6). YmdB was recently reported to regulate the expression and/or activity of a transcriptional regulator SlrR, which in turn affects the activity of both σ^D and the regulator of biofilm formation, SinR, and likely indirectly modulates autolysin activity (19). Finally, TagA is a key enzyme in the synthesis of teichoic acids, a major component of the cell wall (18, 57). The second and third groups of insertions are not directly linked to cell wall homeostasis. The second group, including *kinD, spo0A, qoxAB* and *ssrA* insertions, had relatively mild effects on CEF resistance. Further studies are needed to define the mechanisms of these effects, but in several cases the mutant strains grew more slowly than WT strain under our experimental conditions and this may contribute to their increased ß-lactam resistance (Table 4.3).

Here, we focus on the third group of mutations (*gdpP*, *rsiX*, and *clpP*) for further analysis since they resulted in strong CEF resistance and have been linked to

σ^M and its regulon members. We recovered 11 independent insertions within the 1980 bp coding sequence of *gdpP* (formerly *yybT*). GdpP is a transmembrane protein containing three functional domains: a heme-binding PAS domain, a degenerate GGDEF domain, and a DHH/DHHA1 phosphodiesterase (PDE) domain (71, 72). The *S. aureus* ortholog has recently been renamed GdpP to indicate that it is a GGDEF domain protein containing phosphodiesterase (17) and we therefore adopt this same designation for *B. subtilis*. RsiX is the anti-σ factor cognate for σ^X. We hypothesized that the *rsiX*::Tn7 insertion increased ß-lactam resistance by upregulation of σ^X. Tn7 insertions in *clpP* led to the highest level of CEF resistance observed in this study (Table 4.3). ClpP is a component of the Clp protease. In *B. subtilis*, the ClpP proteolytic core can pair with any of the three Clp ATPases (ClpX, ClpC and ClpE) and form a large hetero-oligomeric Clp protease. Clp protease recognizes and degrades a wide range of proteins, including non-native proteins and stress response regulators, and it is therefore involved in multiple cell development and stress response pathways (24). Here, we present evidence that these three insertion mutations affect three inter-related pathways for CEF resistance (Figure 4.1).

The role of σ^X in CEF resistance is in part through regulation of *abh* and *spx*. We hypothesized that the Tn7 insertion in *rsiX* restored CEF resistance by up-regulation of σ^X which, as noted above, plays a secondary role in CEF resistance that becomes important in the absence of σ^M (Figure 4.1). As predicted, epistasis experiments

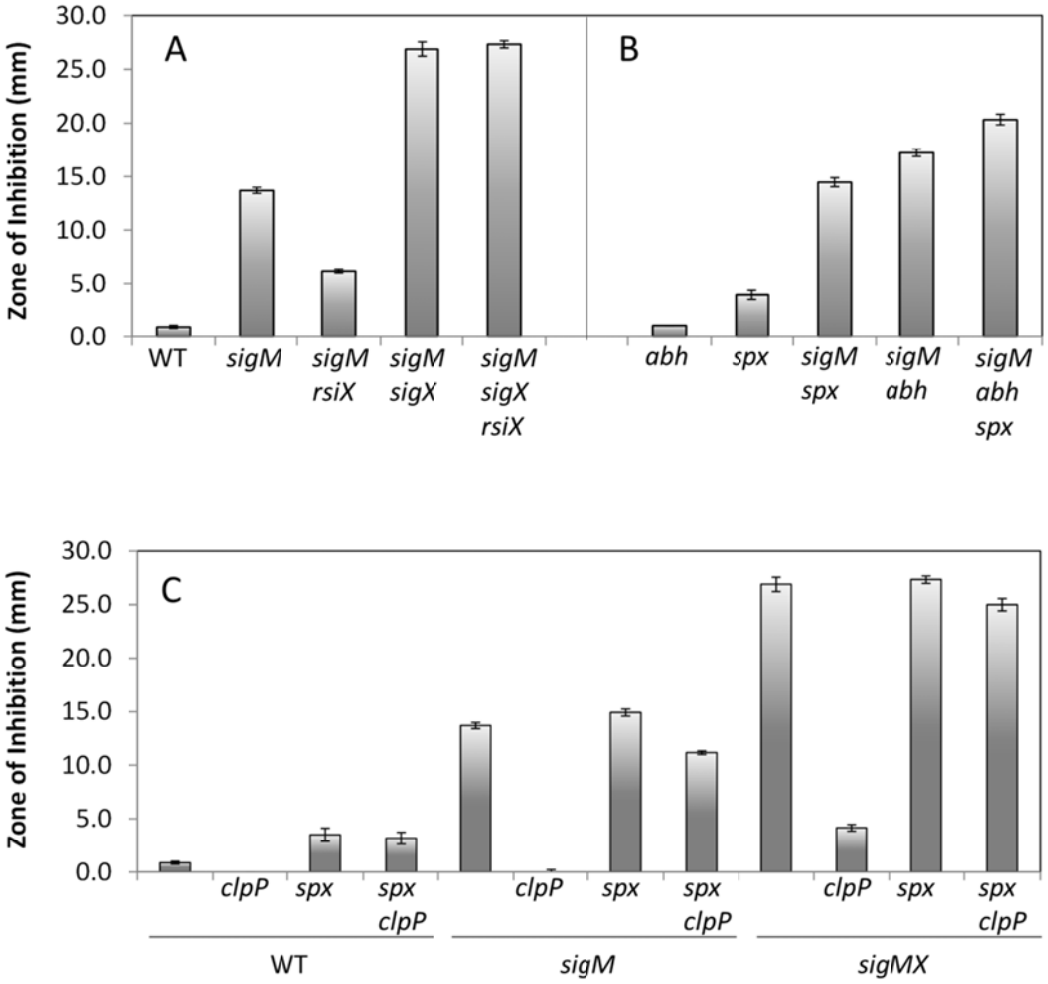

Figure 4.4. The role of *rsiX* and *clpP* mutations in CEF resistance. **(A)** Increased CEF resistance due to an *rsiX* null mutation depends on σ^X. **(B)** *abh* and *spx* mutations are additive to *sigM* with respect to CEF sensitivity. **(C)** Increased CEF resistance due to a *clpP* null mutation depends on Spx in all three strain backgrounds. For ease of comparison, some strains are shown in multiple panels. The susceptibility of each strain was tested using disk diffusion assays with 6 μg CEF. The means and SE at least from 3 biological replicates and 2 independent experiments are reported.

indicated that σ^X is downstream of RsiX: a *sigM sigX rsiX* strain was as sensitive to CEF as the *sigM sigX* strain (Figure 4.4A).

Since the effect of *sigX* on CEF resistance is greatly enhanced in a *sigM* mutant background (Figures. 4.2 and 4.4A), we hypothesized that the relevant genes involved in CEF resistance can be activated by either σ^M or σ^X. The regulons of σ^M and σ^X have been characterized, and several target promoters have been defined that are activated by both ECF σ factors (6, 21). We chose six such target operons (*abh*, *spx*, *dltABCDE*, *lytR*, *yceCDEF*, and *bcrC*) for further analysis. In a WT background, only the *spx* null mutant showed increased CEF susceptibility. When introduced into the *sigM* null mutant, the *abh* and *spx* mutations both increased CEF sensitivity (Figure 4.4B). The *abh* and *spx* CEF sensitive phenotypes in both *sigM* and *sigMX* background can be complemented using IPTG-inducible *abh* or *spx* alleles, respectively (Figures S4.1 and S4.2). These results suggest that *spx* and *abh* can account for at least part of the role of σ^X in CEF resistance. We also defined the MIC of single and multiple mutant strains of *sigM*, *abh,* and *spx*. Although their differences in CEF susceptibility are readily detected in the disk diffusion assay (Figure 4. 4A and B), mutant strains of *sigMX*, *sigM abh*, *sigM abh spx* have the same MIC of 0.03 μg/ml when measured in liquid medium (Table S4.3). We therefore focus here on the differences observed on solid medium.

Abh is a paralog of AbrB and together these two transition state regulators regulate biofilm formation, autolysin activity, and antibiotic production and resistance (52, 59, 60, 82). The transcription of *abh* is dependent on σ^X and σ^M, with σ^X being the major regulator (39, 52). Recently, an *abh* mutant was shown to be sensitive to β-

lactam antibiotics ampicillin, carbenicillin, and cephalexin (59). Resistance to ampicillin was restored by inducing the expression of the transcriptional regulator *slrR*, or by inactivating genes encoding major autolysins (*lytC* and *lytF* encoding amidase and DL-endopeptidase, respectively) (59). These results support a model (Figure 4.1) in which Abh indirectly activates the expression of SlrR (60). SlrR forms a heteromeric complex with SinR which represses both the *lytABC* and *lytF* operons (12). Thus, σ^X and σ^M play partially redundant roles in ß-lactam resistance by activating Abh, which in turn activates SlrR to enable repression of autolytic enzymes.

Accumulation of Spx can increase CEF resistance. Next, we investigated the genetic basis for increased CEF resistance in the *clpP* mutant strains. Several of the reported phenotypes of *clpP* mutants have been linked to increased accumulation of Spx (62, 63), a global regulator of oxidative stress responses (92). There are at least four promoters that control expression of Spx, including one activated by σ^M and σ^X (21). Previously, we determined that *spx* was transcriptionally activated ~3-fold by vancomycin in a σ^M-dependent manner (21) and a similar induction was also reported by Jervis *et al.* using *lacZ*-fusions (41). Other cell wall antibiotics also induce the Spx regulon including amoxicillin (21, 40) and enduracidin (75).

β-lactam antibiotics trigger the production of ROS (32, 46), and Spx is known to protect against oxidative stress (15, 63, 66, 90). We therefore hypothesized that the upregulation of Spx by σ^M might provide a pathway by which ECF σ factors contribute to antibiotic resistance (Figure 4.1). Indeed, in *S. aureus* mutation of the adaptor protein YjbH was recently found to lead to a modest increase in β-lactam resistance which may be due to stabilization of Spx (29).

We used a genetic approach to explore the role of ClpP and Spx in ß-lactam resistance. As noted above (Table 4.3), a *clpP*::Tn7 mutation greatly increased CEF resistance in the *sigM gdpP* mutant strain (HB10264). The *clpP* null mutation also increased CEF resistance in WT and null mutant strains of *sigM* and both *sigM* and *sigX* (Figure 4.4C). Spx is a ClpXP substrate (64). The *spx* mutation masked the effect of *clpP* in the WT, *sigM,* and *sigM sigX* strain backgrounds (Figure 4.4C). These epistasis results imply that *spx* is downstream of *clpP* in the CEF resistance pathway and is the major ClpP substrate that plays a role in ß-lactam resistance. Thus, we predict that the major impact of the *clpP* mutation is to enhance accumulation of Spx in the cell. To test this idea, an IPTG inducible copy of *spx* or spx^{DD} (a Clp protease insensitive variant; (63)) was introduced in the *sigM* and *sigM sigX* mutant strains. An increase in CEF resistance was observed when either *spx* or spx^{DD} was induced (although the effect was much more dramatic with the protease-insensitive allele), suggesting that the accumulation of Spx can increase resistance to CEF in *B. subtilis* (Figure S4.2). In addition, we performed disk diffusion assays with strains lacking either *clpX* or *clpC* (Figure S4.3). Deletion of *clpX* can strongly increase CEF resistance in both strain backgrounds of WT and *sigM* mutant, while deletion of *clpC* only showed minor effect. This result is consistent with the major role of ClpP in CEF resistance being the ClpXP-dependent degradation of Spx.

We also note that the effect of the *clpP* mutation may not be limited to enhancing accumulation of Spx, since mutation of *clpP* also led to a small increase in CEF resistance in a *spx* mutant background. This effect was most notable in strains mutant for *sigM* or *sigM* and *sigX* (Figure 4.4C). A small increased in CEF resistance

was also found with a *clpC* mutant (Figure S4.3). Therefore, we suggest that there are other ClpP protease substrates that also contribute, albeit modestly, to CEF resistance. One candidate is SlrR which, as noted above, has been implicated in the down-regulation of autolysins and is subjected to degradation by ClpCP (11) (Figure 4.1). A second candidate and a ClpCP-degraded substrate is MurAA. MurAA is a UDP-N-acetylglucosamine 1-carboxyvinyltransferase, which catalyzes the first committed step in PG biosynthesis (44).

c-di-AMP as an emerging second messenger found in Bacteria. The most frequent insertions recovered in our selection (Table 4.3) were in *gdpP* and inactivate a PDE known to degrade c-di-AMP, an emerging second messenger found in Bacteria and likely in Archaea (73). c-di-AMP was discovered as a metabolite bound in the crystal structure of DisA which catalyzes its synthesis from ATP (88). DisA was initially characterized as a DNA integrity scanning protein that signals the integrity of the DNA and thereby enables sporulation to proceed (3). This led to a model in which the DisA diadenylate cyclase (DAC; DUF147 domain) signals chromosome integrity: DAC activity can be strongly inhibited by binding of DisA to branched chain nucleic acid structures that might form as recombination intermediates.

DisA is the only confirmed c-di-AMP cyclase (DAC) in *B. subtilis* (65, 88). However, *B. subtilis* encodes two additional candidate DAC proteins (containing DUF147 domains): YbbP and YojJ (73). The DisA DAC domain is linked to a helix-hairpin-helix non-specific DNA-binding domain which allows DAC activity to be regulated by DNA integrity. In contrast, YbbP is predicted to be membrane-localized and YojJ cytosolic, but little is known of how their activities might be regulated. Of

relevance to the present study, transcription of *disA* is regulated by both σ^A and σ^M (21).

The level of c-di-AMP in the cell is controlled by both its rate of synthesis by DAC and its degradation by a c-di-AMP specific phosphodiesterase (PDE) (Figure 4.1). *B. subtilis* GdpP (formerly YybT) is a c-di-AMP PDE *in vitro* (71, 72) and *in vivo* (65). In vegetatively growing *B. subtilis*, 1.7 μM c-di-AMP was measured which increased, in a DisA-dependent manner, to near 5 μM early during sporulation. A *gdpP* deletion strain of *B. subtilis* was shown to have a >4-fold increase in c-di-AMP levels in early sporulating cells (65). Similarly, a ~15 fold increase was observed with a *gdpP* mutation in *S. aureus* (from 2.8 μM to 42.9 μM). In *S. aureus*, elevated levels of c-di-AMP suppress the growth defects associated with an inability to synthesize LTA and alter both autolysin expression and the level of PG crosslinking (17).

In *B. subtilis*, the synthesis and degradation of c-di-AMP is correlated with ß-lactam resistance. GdpP is a transmembrane protein with three functional domains: a heme-binding PAS domain, a degenerate GGDEF domain, and a DHH/DHHA1 PDE domain (71, 72). In accordance with the emerging model of c-di-AMP as a signal molecule, we hypothesized that it was the loss of GdpP PDE activity that conferred CEF resistance. We therefore complemented the *sigM gdpP* strain with an IPTG-inducible GdpP, a truncated GdpP lacking the DHH/DHHA1 domain ($GdpP_{1-303}$), or a mutated GdpP ($GdpP_{D420A}$) carrying a single amino acid substitution which abolishes PDE activity (72). Induction of WT GdpP conferred an extreme CEF sensitivity (Figure 4.5). In contrast, neither of the mutant GdpP proteins increased sensitivity to CEF (Figure 4.5), suggesting that it is the PDE activity that affects CEF sensitivity.

One consequence of antibiotic stress is the activation of σ^M which leads to elevated expression of the DisA DAC. We therefore hypothesized that a *sigM* null mutant might have decreased c-di-AMP levels that could be compensated by mutation of GdpP, the c-di-AMP degrading PDE. Indeed, a *disA* deletion mutant displayed a small but reproducible increase in sensitivity to CEF, with an MIC of 3 μg/ml compared to 4 μg/ml for WT (Table S4.3). This is consistent with the recent report that DisA accounts for perhaps 50% of the c-di-AMP present in cells as monitored early in sporulation (65). As expected, the induction of GdpP in the *disA* mutant led to a large increase in CEF susceptibility (Figure 4.6), consistent with the notion that even *disA* cells contain substantial c-di-AMP that contributes to CEF resistance. This suggests that *B. subtilis* contains at least one additional DAC, presumably encoded by either or both the DAC-domain containing proteins YbbP and YojJ.

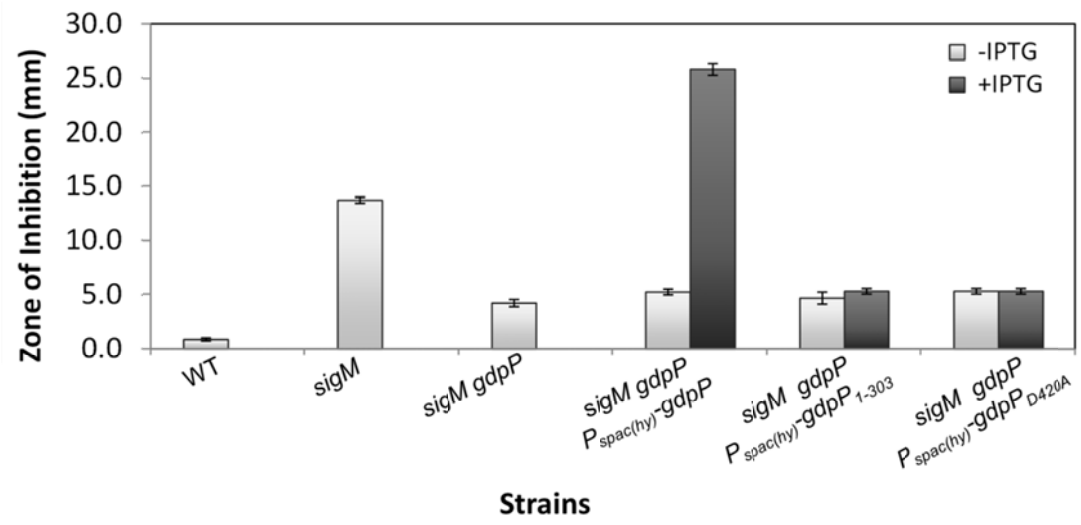

Figure 4.5. The DHH/DHHA1 domain of GdpP is required to restore CEF sensitivity to the resistant strain *sigM gdpP*. Disk diffusion tests were performed with 6 μg CEF. The means and SE based on 3 biological replicates and 2 independent experiments are shown. 1 mM IPTG was added where indicated.

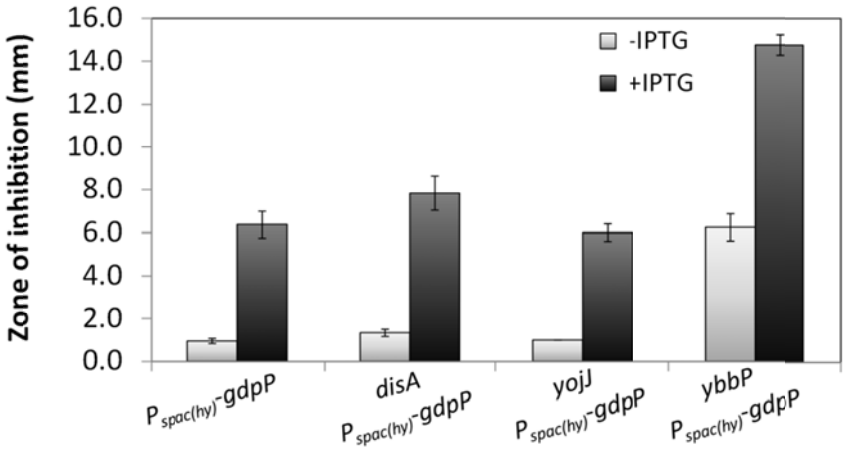

Figure 4.6. Induction of the GdpP PDE increases CEF sensitivity in WT and cells individual DAC enzymes. Disk diffusion tests were performed with 6 µg CEF. The means and SE based on 3 biological replicates and 2 independent experiments are reported. 1 mM IPTG was added where indicated.

c-di-AMP is essential for cell growth. To gain insights into the relative contributions of *disA, ybbP,* and *yojJ* to c-di-AMP synthesis we mutated each of these loci individually and in combination. Deletion of *ybbP* resulted in the highest CEF sensitivity (as seen in the uninduced sample in Figure 4.6, and MIC of 1 μg/ml, Table S4.3). Deletion of *yojJ*, however, had no effect. Induction of GdpP increased CEF sensitivity in all three DAC mutant backgrounds (Figure 4.6). We conclude that YbbP is the major DAC contributing to intrinsic β-lactam resistance in growing cells, and that both synthesis and degradation of c-di-AMP affects CEF resistance. This result is consistent with the recent suggestion that DisA functions primarily in early sporulation, with a comparatively minor contribution in (unstressed) vegetative phase cells (65). It is interesting to note that YbbP and GdpP are both membrane-localized, although the signals that might control their synthesis and activity are unknown.

The expression of YbbP is poorly characterized, but it is noteworthy that it is encoded immediately downstream of the *sigW-rsiW* operon and it may be, in part, activated by σ^W. However, σ^W has no effect in CEF resistance in our *B. subtilis* WT strain background (Figure 4.2). We therefore asked whether σ^M or σ^X have a role in regulating *ybbP*. Multiple null mutants of *sigM*, *sigX*, and *ybbP* were constructed and tested for their susceptibilities to CEF. The mutation in *ybbP* is clearly additive to both *sigM* and *sigX* mutations (Figure S4.4). In addition, the transcriptional start site of *ybbP* was mapped to 72 bp upstream of its start codon using 5'RACE. A σ^A promoter is present upstream of the assigned start site (TTCACTtgctaaatcgaaatgtggTATAATgggctcG; upper case letters indicate the -35, -10, +1 regions, respectively). Together, these results suggest that *ybbP* is not part of

the σ^M or σ^X regulatory pathways.

We next sought to construct double and triple null mutants of *disA, ybbP,* and *yojJ*. A *disA ybbP* double mutant strain could not be obtained, suggesting that this combination of mutations is lethal, whereas double mutants of *disA yojJ* and *ybbP yojJ* were viable. We conclude that c-di-AMP is essential for viability and that the basal level of expression of either DisA or YbbP is sufficient to support growth. An essential role for DAC proteins has also been suggested in *Listeria monocytogenes* since it was impossible to disrupt the single DAC encoding gene in this organism (89). Similarly, DAC genes were identified in screens for essential genes in *Mycoplasma* spp., *Streptococcus pneumoniae,* and *S. aureus* (14, 25, 28, 81).

To determine whether all three DAC proteins (DisA,YbbP, YojJ) are active and could support growth, we integrated an IPTG-inducible copy of each gene into a *ybbP* null mutant and then attempted to introduce a *disA* null mutation by chromosomal transformation. Indeed, a *disA ybbP* double mutant could be obtained when any one of the three genes (*disA, ybbP, or yoj*) was induced (Figure 4.7A). This strategy also allowed construction of IPTG-dependent *disA ybbP yojJ* triple mutant strains in which growth could be supported by any one of three DAC-encoding genes. We note that the $P_{spac(hy)}$ promoter used in this work is slightly leaky and, as a result, the *disA ybbP* $P_{spac(hy)}$*-disA* strain was able to grow even in the absence of IPTG. However, the *disA ybbP* $P_{spac(hy)}$*-yojJ* strain grew slowly and the *disA ybbP* $P_{spac(hy)}$*-ybbP* was unable to grow unless at least 50 μM IPTG was present (data not shown). These results suggest that all three of these putative DAC proteins are biologically active and able to support growth when expressed.

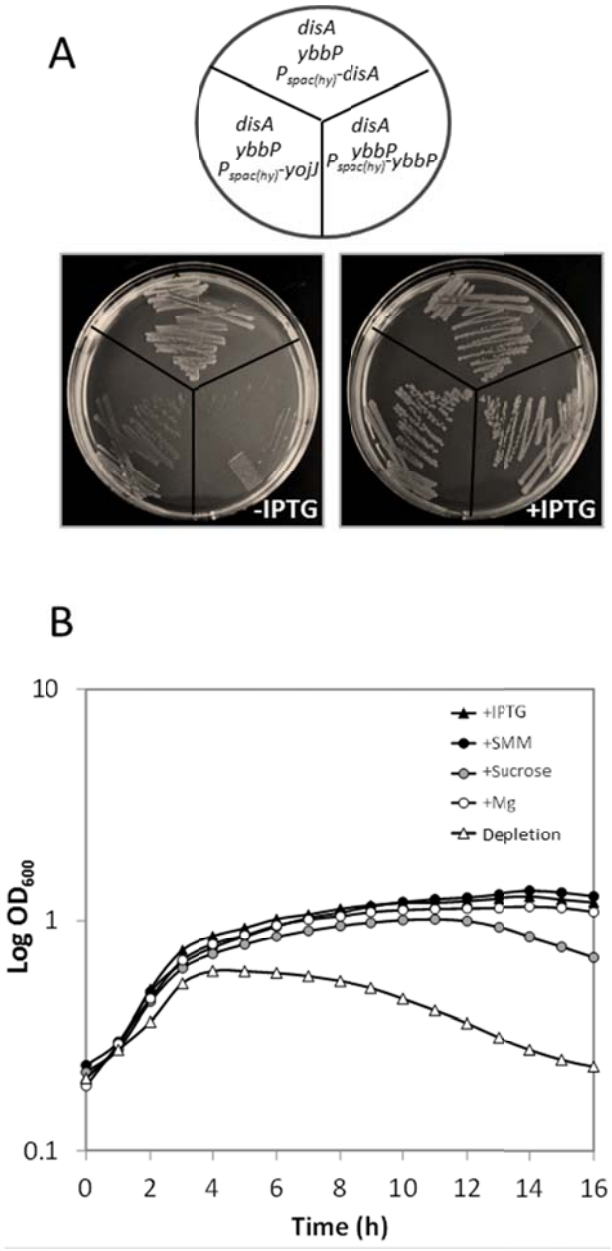

Figure 4.7. *disA* and *ybbP* are synthetically lethal. **(A)** Strains of *disA ybbP* harboring IPTG inducible *disA*, *ybbP* or *yojJ* were grown on MH agar plates supplemented with or without 1 mM IPTG. **(B)** Depletion of *ybbP* in strain *disA ybbP* $P_{spac(hy)}$-*ybbP* results in cell lysis. Cells were grown in presence of 1 mM IPTG to mid-log phase, washed, resuspended in fresh MH medium alone or with additional 1 mM IPTG, SMM, 10% sucrose or 10 mM Mg, and returned to 37°C incubation with vigorous shaking. Growth was measured by OD_{600} using a Bioscreen incubator. Ten biological replicates were tested, and showed similar growth pattern. Growth curves from one representative experiment are shown.

The essential role of c-di-AMP is linked to PG homeostasis. Since a reduced level of c-di-AMP is linked to high CEF sensitivity, we tested whether c-di-AMP is involved in cell wall homeostasis. Depletion of c-di-AMP in strain *disA ybbP* $P_{spac(hy)}$-*ybbP* by growth in the absence of inducer IPTG led to cell lysis as monitored both by following optical density (Figure 4.7B) and by light microscopy (Figure S4.5). The lysis phenotype can be suppressed either by the presence of IPTG (inducing the expression of *ybbP*), or by supplementation of the growth medium with SMM (sucrose, $MgSO_4$ and maleic acid), sucrose, or $MgSO_4$. SMM has been used previously to stabilize protoplasts and support the growth of cell wall-free L-form cells (13, 47). Similarly, sucrose likely functions as an osmotic protectant, and Mg^{2+} has been shown to restore growth and WT morphology of many PG defective mutants including single mutants of *ponA, rodA, mreB, mreC, mreD, mbl* and a double mutant of *pbpAH* (22, 42, 43, 48, 61, 79). This is reminiscent of recent results from Corrigan *et al.* (17) who showed that osmotic protectants support the growth of a LTA deficient mutant of *S. aureus* and that this requirement can be bypassed by a *gdpP* mutation. The *S. aureus gdpP* mutant displayed an increase in both c-di-AMP and PG cross-linking. Collectively, these results suggest that c-di-AMP plays an essential role in PG homeostasis (Figure 4.1).

σ^M and c-di-AMP are involved in resistance to other cell wall antibiotics. We next tested whether c-di-AMP is involved in resistance to other antibiotics. Induction of GdpP in strain *sigM gdpP* $P_{spac(hy)}$-*gdpP* leads to high sensitivity to aztreonam, cefixime, and moenomycin in addition to CEF as monitored using disk diffusion assays (Figure 4.8). Cefixime is a third generation cephalosporin, aztreonam is a

monobactam, and moenomycin is a glycolipid. As β-lactams, cefixime and aztreonam target PBP transpeptidases. Moenomycin, on the other hand, targets the TG activity of HMW-PBPs (50). Although aztreonam is generally found to have poor activity against Gram positive bacteria (26, 31) we observed using bocillin-FL labeling that aztreonam can derivatize PBP 1, 2c, and 4 in *B. subtilis* (Figure 4.3). As also noted for CEF, mutation of *sigM* converts *B. subtilis* from an aztreonam non-susceptible to a susceptible strain, and this susceptibility is modulated by *gdpP* (Figure 4.8). Thus, the function of c-di-AMP is not limited to CEF resistance, as would be expected if it functions to support balanced cell wall synthesis.

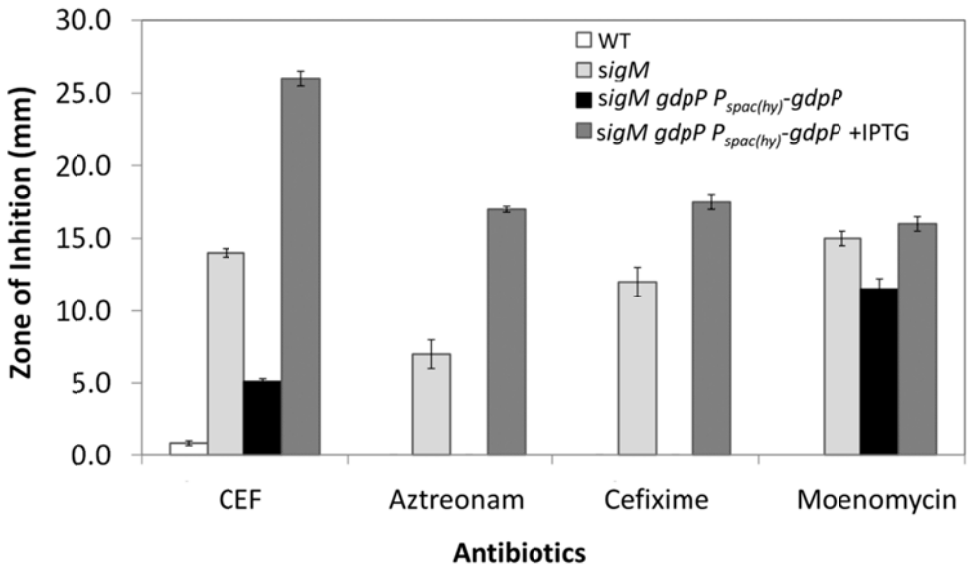

Figure 4.8. c-di-AMP is involved in intrinsic resistance to other cell wall antibiotics. Disk diffusion tests were performed with CEF (6 µg), aztreonam (30 µg), cefixime (5 µg) and moenomycin (50 µg). The means and SE from biological triplicates are shown. Note that no zone of inhibition could be detected with aztreonam or cefixime in WT and the uninduced s*igM gdpP* $P_{spac(hy)}$*-gdpP* strain.

A model for the role of ECF σ factors in ß-lactam resistance. The genetic analyses presented herein lead to an integrated model in which the ECF σ factors σ^M and σ^X contribute to ß-lactam resistance by the antibiotic-inducible activation of regulatory proteins that affect three distinct pathways (Figure 4.1). *B. subtilis* PG is a dynamic structure, which is continuously synthesized, modified, and hydrolyzed. It is notable that σ^M-activated promoters have been previously mapped preceding several genes involved in PG synthesis (including *mreB, bcrC, divIB, divIC, ddl, murB, murF, rodA, pbpX,* and *ponA*), one of the four paralogous LTA synthases (*yfnI*), and cell wall modification enzymes (*dltABCDE*) (21). Thus, σ^M appears to function to positively regulate cell wall assembly and structure in response to antibiotic stress. β-lactam antibiotics inhibit the TP activity of PBPs and thereby inhibit glycan strand cross-linking. This inhibition disrupts the balance between PG synthesis and hydrolysis and endogenous autolysins trigger cell lysis. In addition, ß-lactams trigger ROS formation and cell death. Both autolysin-dependent and independent mechanisms contribute to the bactericidal effect (20, 46).

ECF σ factors counteract the effects of ß-lactams by activating at least three distinct pathways (Figure 4.1). First, σ^M contributes to the expression of one of three c-di-AMP synthases (DisA). The cellular level of c-di-AMP is regulated by both DAC synthases (DisA, YbbP and YojJ) and the cognate PDE (GdpP). At least one DAC is required for cell growth, indicating an essential role of c-di-AMP. The cell lysis phenotype of our DAC depletion strain together with the recent report from Corrigan *et al.* (17) suggest a positive link between c-di-AMP and PG cross-linking. However, the role of c-di-AMP may be not limited to cross-linking, since c-di-AMP also

modulates susceptibility to moenomycin, which targets the TG domain of PBP and thereby inhibits the polymerization of the PG glycan strands.

Second, ECF σ factors affect the expression and regulation of autolysins. Both σ^M and σ^X activate the transcription of *abh*, whose product indirectly activates the expression of SlrR, which directly represses expression of LytC and LytF (12, 52, 59). Another autolytic endopeptidase (LytE) was identified by Tn7 mutagenesis as a contributor to ß-lactam susceptibility. These findings support the notion that preventing autolysis can increase ß-lactam resistance.

Third, our analysis of the ß-lactam resistance phenotype of a *clpP* null mutant identified Spx, a regulator of pathways that protect the cell against ROS (92), as a contributor to ß-lactam resistance. The *clpP* mutant strain may also have elevated levels of SlrR, a known inhibitor of autolysin expression (11). Although the model we have developed here (Figure 4.1) is already quite complex, it certainly underestimates the true complexity of the adaptive responses mediated by ECF σ factors and other regulators that conspire to protect cells against antibiotics and other chemical insults.

4.4 Acknowledgments

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The supplementary information (Table S3.1) can be found at the end of this chapter.

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4.5 Supplementary Information

Table S4.1. Oligos used in this study

#	Name	Sequence[1]
5244	yybT-for(xbaI)	GAGTCTAGA CATGGTGGGGAGTGATAGAAATGC
5245	yybT-rev(bglII)	GAGAGATCTTCATCTCTGTACGCCTCCCT
5258	yybT(1-303aa) rev(bglII)	GAGAGATCTTTAGCCGTTTGGCAGCTTAATG
5249	disA for(xmaI)	GAGCCCGGGTACTTCATTAGGAGGATAATAGATG
5250	disA rev(ClaI)	GAGATCGATTCATAAGGTTTTAACCGAAATCA
5252	ybbP for(XmaI)	GAGCCCGGGAATCTTGGAGGACGAGGAAATG
5253	ybbP rev(ClaI)	GAGATCGATAGCGGTTGTTTAAGAATTTATCCA
5255	yojJ for(xmaI)	GAGCCCGGGTTCGTGAAAAGTTGGAAATTTAAACAGGAG
5256	yojJ rev(ClaI)	GAGATCGATTGTCTCATGATAGGATTCTTAATCAG
5293	yybT D420A up-rev	TGTGTAGCAACGATCACAAGCAGTGT
5294	yybT D420A do-for	ACACTGCTTGTGATCGTTGCTACACATAAGCCGTCACTCGT
5584	ybbP-rev-GSP3	GAACAAGCACGATGACTACA
5585	ybbP-rev-GSP4	TACCAAACAAGGAGAATATCA

[1] The endonuclease digestion sites are underlined.

Table S4.2. Strains used in the supporting information.

Strain	Genotype	Reference/ Construction
JH642	*trpC2 pheA1*	Lab strain
PY79	SPβ-cured prototroph strain	Lab strain
BZH73	JH642 *abh::kan amyE::P_{spac}-abh cat thrC::sunA'-lacZ spc*	(82)
HB10158	168 *amyE::P_{spac}-abh cat*	chrDNA of BZH73 --> 168
HB10159	168 *sigM::tet amyE::P_{spac}-abh cat*	chrDNA of HB10158 --> HB10016
HB15808	168 *sigM::kan abh::spc*	chrDNA of HB10131 --> HB10216
HB15809	168 *sigM::tet abh::spc amyE:: P_{spac}-abh cat*	chrDNA of HB10131 --> HB10159
HB15810	168 *sigM::tet sigX::kan amyE:: P_{spac}-abh cat*	chrDNA of HB10103 --> HB10159
ORB4271	JH642 *amyE:: $P_{spank(hy)}$-spx spc*	(63)
ORB4342	JH642 *amyE: $P_{spank(hy)}$-spx^{DD} spc*	(63)
HB10392	168 *amyE:: $P_{spank(hy)}$-spx spc*	chrDNA of ORB4271 --> 168
HB10393	168 *amyE:: $P_{spank(hy)}$-spx^{DD} spc*	chrDNA of ORB4342 --> 168
HB10394	168 *sigM::kan amyE:: $P_{spank(hy)}$-spx spc*	chrDNA of ORB4271 --> HB10216
HB10395	168 *sigM::kan amyE: $P_{spank(hy)}$-spx^{DD} spc*	chrDNA of ORB4342 --> HB10216
HB15817	168 *sigM::kan spx::mls amyE:: $P_{spank(hy)}$-spx spc*	chrDNA of HB10348 --> HB10394
HB15818	168 *sigM::kan spx::mls amyE: $P_{spank(hy)}$-spx^{DD} spc*	chrDNA of HB10348 --> HB10395
HB15821	168 *sigM::tet sigX::kan amyE:: $P_{spank(hy)}$-spx spc*	chrDNA of HB10392 --> HB10113
HB15822	168 *sigM::tet sigX::kan amyE: $P_{spank(hy)}$-spx^{DD} spc*	chrDNA of HB10393 --> HB10113
HB10372	168 *sigM::kan disA::spc*	chrDNA of HB10216 --> HB10353
HB10375	168 *sigM::kan ybbP::tet*	chrDNA of HB10216 --> HB10355
HB10390	168 *sigM::kan ybbP::tet sigX::spc*	chrDNA of HB7007 --> HB10375

Table S4.2. (Continued)

Strain	Genotype	Reference/ Construction
RL2774	PY79 *clpC::tet*	(11)
RL2173	PY79 *clpX::spc*	Win Chai
HB15839	168 *clpC::tet*	chrDNA RL2774 --> 168
HB15840	168 *sigM::kan clpC::tet*	chrDNA RL2774 --> HB10216
HB15841	168 *clpX::spc*	chrDNA RL2173-->168
HB15842	168 *sigM::kan clpX::spc*	chrDNA RL2173--> HB10216

Table S4.3. MIC values of strain 168 and its derivative mutants.

Strain #	Genotype	MIC (CEF, µg/ml)
168	WT	4
HB10216	*sigM::kan*	0.06
HB10113	*sigM::tet sigX::kan*	0.03
HB10131	*abh::spc*	2
HB10328	*spx::spc*	3
HB15808	*sigM::kan abh::spc*	0.03
HB10329	*sigM::kan spx::mls*	0.06
HB15811	*sigM::kan abh::spc spx::mls*	0.03
HB10353	*disA::spc*	3
HB10334	*ybbP::tet*	1
HB10335	*yojJ::kan*	4

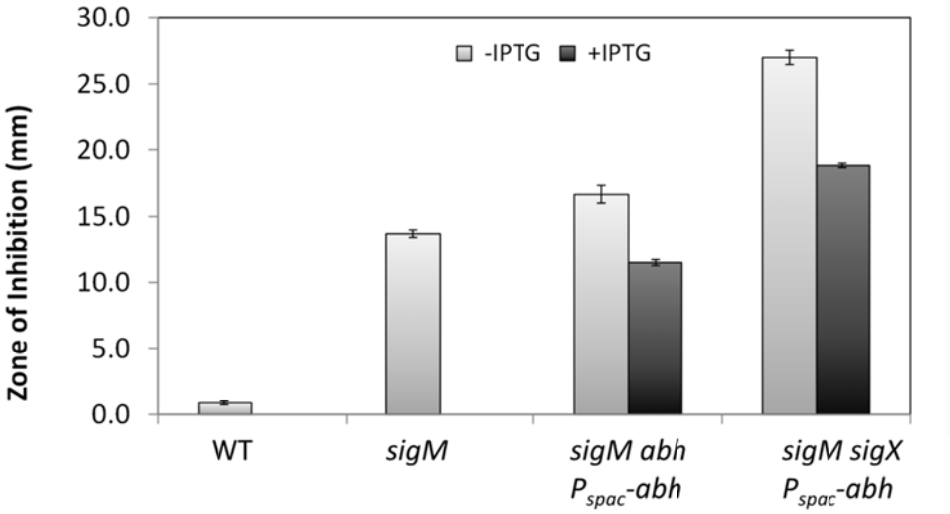

Figure S4.1. Complementation test of *abh*. Disk diffusion tests were performed with 6 μg CEF. Averages and SE based on three biological replicates and two independent experiments are shown. 1 mM IPTG was added at where indicated.

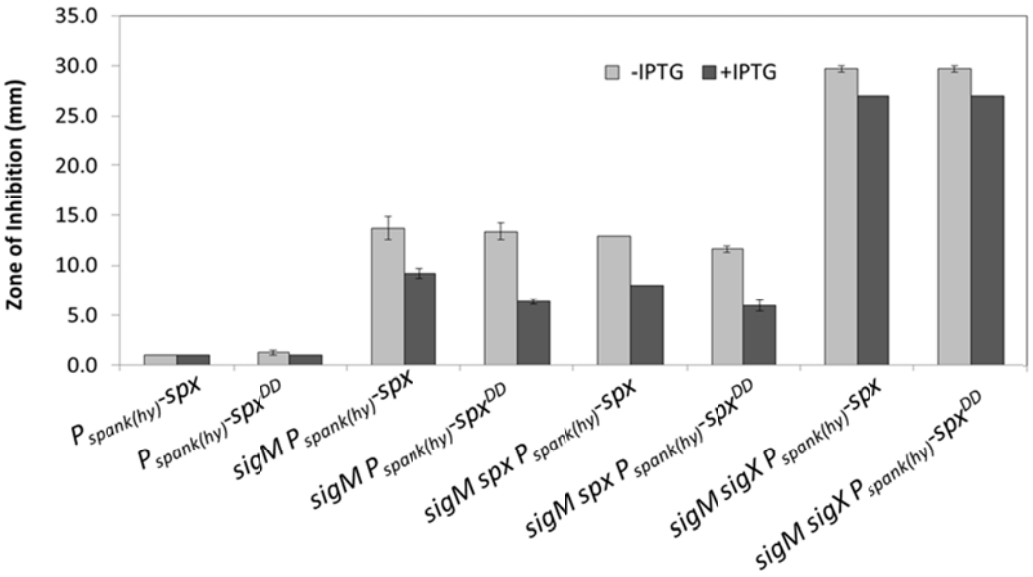

Figure S4.2. Complementation test of *spx*. Disk diffusion tests were performed with 6 μg CEF. Averages and SE based on three biological replicates and two independent experiments are shown. 1 mM IPTG was used to induce $P_{spank(hy)}$-*spx*, and 0.01 mM IPTG were used to induce $P_{spank(hy)}$-spx^{DD}. Note that Spx is a substrate of ClpXP protease, and thereby it can not be accumulated to a high level even in the presence of 1 mM IPTG. Spx^{DD} can not be degraded by ClpXP, but inducing $P_{spank(hy)}$-spx^{DD} with more than 0.01 mM IPTG is lethal.

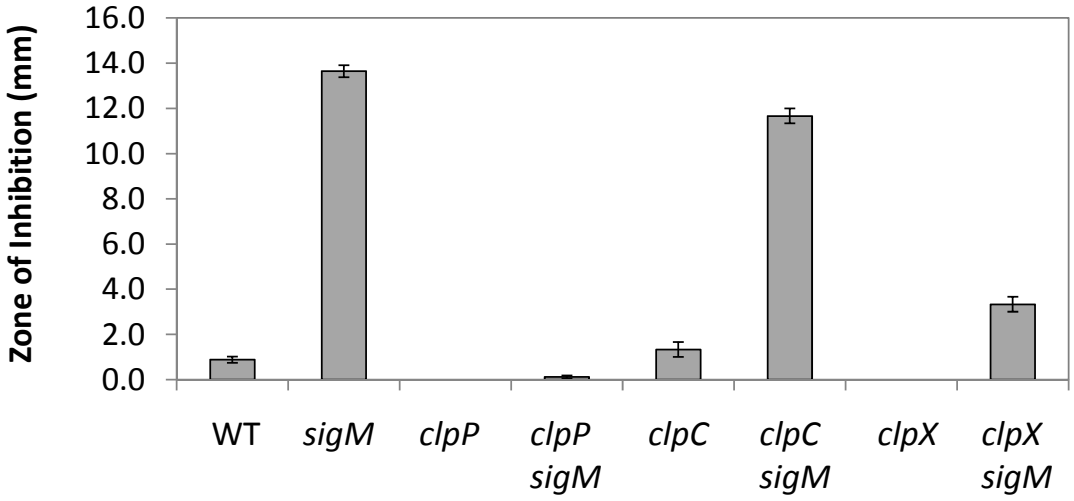

Figure S4.3. Disk diffusion tests with *clpP*, *clpC* and *clpX* mutant in the backgrounds of strain WT and *sigM* mutant and 6 μg CEF. Averages and SE based on three biological replicates and two independent experiments are shown.

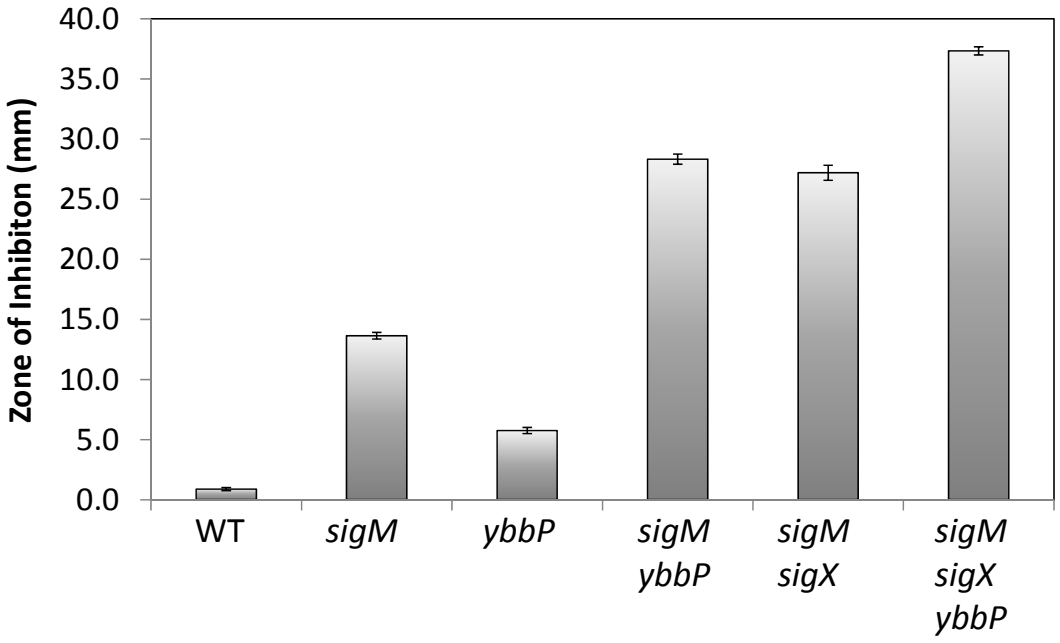

Figure S4.4. *ybbP* is additive to *sigM* and *sigX* in CEF susceptibility. Disk diffusion tests were performed with 6 μg CEF. Averages and SE based on three biological replicates and two independent experiments are shown.

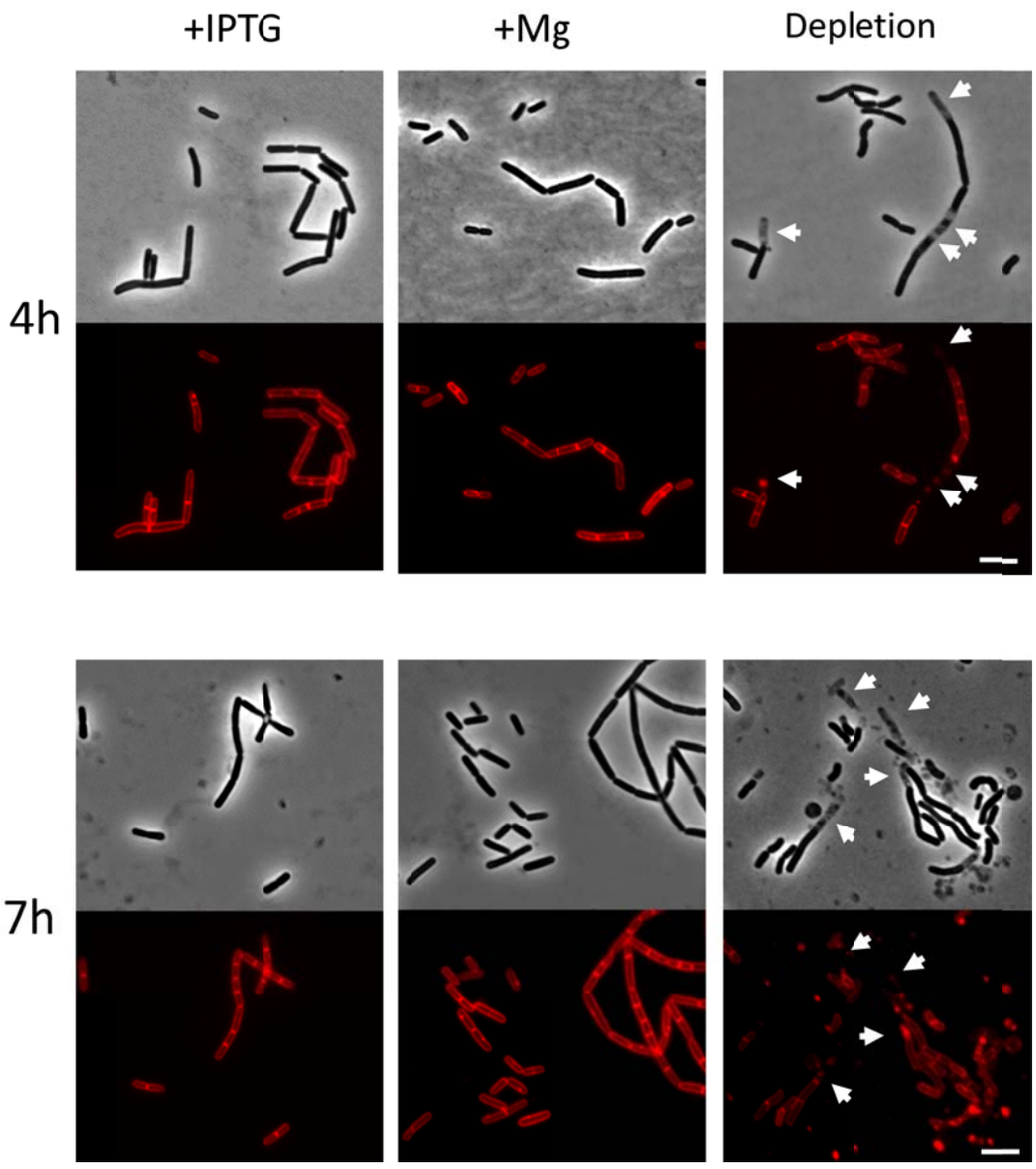

Figure S4.5. Cells observed using phase contrast and fluorescence microscopy. Cells were grown in MH medium supplemented with 1 mM IPTG to mid-log phase, washed, resuspended in fresh MH medium alone, or supplemented with 1 mM IPTG, or with 10 mM $MgSO_4$, and returned to 37°C incubation with vigorous shaking in a Bioscreen incubator. Cells were examined at three time points 2 h (cell morphology was indistinguishable from 4 h; data not shown), 4 h and 7 h. Cell membrane was stained with FM4-64, and colored in red. Arrows indicate lysed cells. Size bar is 10 μm. The corresponding growth curves are shown in Figure 4.7.

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

B. subtilis expresses seven ECF σ factors. In this dissertation, I have primarily characterized strains lacking three of these σ factors (σ^M, σ^W and σ^X) and all seven ECF σ factors (σ^M, σ^W, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC}). I have also described the mechanism by which the ECF σ factors σ^M and σ^X regulate the production of sublancin and resistance to cefuroxime.

In chapter 2, σ^X and σ^M are shown to be involved in sublancin production. These two σ factors regulate the expression of Abh, which is capable of antagonizing repression by AbrB at the *sunA* promoter, resulting in sublancin production. Sublancin is an antibiotic encoded in the lysogenic prophage SPβ. The fact that the chromosome-located σ^M and σ^X can activate the expression of the prophage encoded sublancin, and that σ^W can provide immunity to sublancin(1), suggests an intricate regulatory network of antibiotic production and resistance in *B. subtilis*.

In chapter 3, I compared the transcriptomic profiles and phenotypic traits of the Δ7ECF and ΔMWX strains to the WT strain. Over 80 genes were found to be, at least in part, dependent on ECF σ factors for their transcription. New phenotypes (especially increased susceptibility to compounds targeting at cell envelope) were found to be associated with these two ECF σ factor deletion strains. In addition, the detailed characterization of the Δ7ECF strain in this chapter will facilitate future use of this strain. For example, an ECF σ factor can be introduced back into the Δ7ECF strain, to test its effect in gene regulation. This type of experiment will allow us to

delineate the regulons and function of individual σ factors, without complications due to the presence of other ECF σ factors.

In chapter 4, σ^M and σ^X were shown to be involved in cefuroxime resistance through three pathways: i) a c-di-AMP pathway that mediates PG homeostasis; ii) a Spx pathway that antagonizes ROS damage; and iii) a SlrR pathway that reduces expression of autolysins. An important component of this work is the discovery that the signaling molecule, c-di-AMP, is essential for cell growth and PG homeostasis. In future studies it will be interesting to define those factors regulating both synthesis and degradation of c-di-AMP, and to identify the downstream targets of this signaling molecule.

Of the three synthases of c-di-AMP, the expression and activity of DisA is regulated by σ^M and DNA integrity, respectively (5, 7). There is little known about YbbP and YojJ. YbbP appears to be the major synthase (Chapter 4), and the *ybbP* gene is located downstream of the *sigW-rsiW* operon, with a possible read-through from the *sigW* promoter. This σ^W-dependent promoter could be mutated and tested for its effect on the transcriptional level of *ybbP*, especially in the presence of an antibiotic stress, such as cefuroxime.

GdpP is the only known enzyme that degrades c-di-AMP in *B. subtilis*. *gdpP* is transcribed as part of an operon under control of σ^A. Tiling array experiments revealed an antisense RNA initiated within the *gdpP* coding region (10). We have mapped the start site of this σ^D-dependent transcript, and found that the expression of this transcript can reduce the protein level of GdpP about 3-fold (Data not shown). σ^D is known to activate the expression of autolysins in *B. subtilis* (3, 6). A plausible

explanation for the antisense regulation is that σ^D down-regulates *gdpP* as a mechanism to maintain peptidoglycan (PG) homeostasis: the up-regulated PG biosynthesis (due to the increased level of c-di-AMP) is concomitant with the increased PG degradation (due to the upregulation of autolysins under σ^D control). This idea could be tested using a strain carrying a null mutation of the σ^D promoter yet maintaining the amino acid sequence of GdpP. Presumably this mutant strain will not be able to down-regulate GdpP synthesis when σ^D is activated and this may lead to an imbalance between PG biosynthetic and autolytic functions. In addition, *in vitro* work has shown that the phosphodiesterase activity of YybT protein can be inhibited by two factors, i) the presence of the stringent response alarmone ppGpp (9), and ii) the binding of heme to the PAS domain of GdpP (8). The biological relevance of these two factors, however, needs to be tested *in vivo.* The heme binding domain of GdpP could be mutated and tested whether it could affect the phosphodiesterase activity of GdpP. The level of c-di-AMP and ppGpp can be measured directly, using HPLC followed by LC-MS/MS (4), and radioactive labeling followed by thin layer chromatography (2), respectively. These measurements will allow us to examine whether there is coordination between the concentrations of these two molecules. Mutation of the ppGpp synthase gene *ywaC* could also be constructed and tested for its effect in c-di-AMP level.

Although c-di-AMP is clearly essential for growth, the targets of c-di-AMP are not yet known in any bacterium. It would be of great interest to identify c-di-AMP binding proteins. A feasible method is to use affinity chromatography with a biotinylated c-di-AMP (BioLog) as an affinity probe. Proteins associated with c-di-

AMP can be recovered using streptavidin-sepharose, and further identified by SDS-PAGE followed by tryptic digestion and MS/MS peptide sequencing.

In summary, the work presented in this dissertation revealed an important role for ECF σ factors in antibiotic production and resistance. Future work with ECF σ factors and c-di-AMP can help us understand the complex cell envelope stress response mediated by ECF σ factors, signaling molecules, and the key components of the cell envelope biogenesis machinery.

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